FUNGAL TREATMENTS FOR LIGNOCELLULOSIC BIOCONVERSION

by

Richard Lee Giles

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Approved by:

_____
Dr. Matthew Parrow

_____
Dr. Inna Sokolova

_____
Dr. Gloria Elliott

_____
Dr. Helene Hilger

_____
Dr. Jessica Schlueter

ABSTRACT

RICHARD LEE GILES. Fungal pretreatments for lignocellulosic bioconversion. (Under the direction of DR. MATTHEW W. PARROW)

Four studies were conducted to develop an innovative application of fungal biopulping technologies to improve commercialization of lignocellulosic ethanol biofuel production using sustainable waste products. The first study focused on evaluation and development of fungal pretreatments for lignocellulosic ethanol using waste tulip poplar wood chips. The second study focused on the optimization of fungal pretreatments inoculation methods for laboratory and pilot scale applications. The third study focused on single versus simultaneous species inoculations for reduced pretreatment staging. The fourth study focused on evaluation of North Carolina derived, novel wood decay fungi for two-stage biopulping applications. These studies demonstrate that specific naturally occurring wood decay fungi can be utilized in an innovative two-stage whole organism approach to significantly increase enzymatic hydrolysis yield of soluble glucose. The potential to augment enzymatic hydrolysis is most promising for continued study. Considering the numerous cost and sustainability advantages over current methods due to reduced enzyme load and increased enzymatic hydrolysis yield, novel biopulping technologies could make a significant contribution to the development of environmentally friendly and sustainable commercial lignocellulosic ethanol production.

DEDICATION

This dissertation is dedicated to the memory of my friend and mentor in the study of mycology Dr. Larry F. Grand.

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His advisors, Dr. Matthew Parrow for making this work possible from idea to publication and commercial application, Dr. Gloria Elliott for advice on experimental design and technical guidance, Dr. Inna Sokalova for academic support, and Dr. Helene Hilger for research and professional advisement;

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CHAPTER 1: TWO-STAGE FUNGAL BIOPULPING FOR IMPROVED ENZYMATIC HYDROLYSIS OF WOOD

The following chapter was published in the scientific journal *Bioresource Technology* in 2011. Giles, R.L.; Galloway, E.R.; Elliott, G.D.; Parrow, M.W. Two-stage fungal biopulping for improved enzymatic hydrolysis of wood. Bioresource Technology. 2011, 102, 8011-8016.

Abstract

A novel two-stage, whole organism fungal biopulping method was examined for increasing the yield of enzymatic hydrolysis of wood into soluble glucose for ethanol production. *Liriodendron tulipifera* wood chips were selectively decayed in solid-phase treatments with both white rot (*Ceriporiopsis subvermispora*) and brown rot (*Postia placenta*) fungi in alternative succession. Both fungi were successfully grown on *L. tulipifera* chips and on chips previously colonized by each decay fungus with no inhibitive interaction. Fungal treatments did promote hyphal growth, mass loss, and lignin decay but did not significantly decrease the total carbohydrate content of the wood. Two-stage fungal colonization treatment consisting of C. *subvermispora* followed by *P. placenta* increased the yield of enzymatic hydrolysis by 94%.

Introduction

Lignocellulosic biomass such as wood waste, crop stalks, and grasses are potentially more economical, sustainable sources of biomass for ethanol production than food grains such as corn (Hahn-Hägerdal et al. 2006). However, the abundant sugars contained in these feedstocks are blocked from traditional ethanol-producing fermentation reactions because they typically occur in a complex polymerization of lignin and celluloses that is difficult to hydrolyze into soluble sugars for fermentation (Sun and Cheng 2002, Gupta et al. 2009). Some fungi belonging to the order *Aphyllophorales* naturally decay lignocellulosic biomass, removing lignin and depolymerizing cellulose in the process (Eriksson et al. 1990, Perez et al. 2002). These fungi have already been used on an industrial scale to reduce the mechanical breakdown energy required to pulp wood (i.e. soften through delignification) for paper production (Aktar et al. 1992, Scott et al. 1998), and should also significantly increase the availability of cellulosic sugars for fermentation into ethanol. Fungal species used in paper biopulping belong to a group responsible for a natural wood decay phenomenon called white rot. Most white rot fungi degrade both lignin and celluloses in wood (Eriksson et al. 1990). However, some species of white rot fungi selectively decay lignin and leave cellulose sugars intact (Perez et al. 2002, Choi et al. 2006). These lignin selective white rot fungi have previously been used in papermaking to efficiently delignify lignocellulosic biomass, allowing significantly improved access to cellulose (Scott et al. 1998).

Lignin selective fungi alone, while removing lignin from wood, do not break down the anamorphous and crystalline regions of celluloses into fermentable sugars (Eriksson et al. 1990). A second hydrolysis step must be used to break down the cellulose

before use in fermentation, and this is the significant, cost prohibitive and limiting step in commercial production of ethanol from wood (Sun and Cheng 2002, Hahn-Hägerdal et al. 2006). Efficient industrial cellulose hydrolysis requires synergistic combinations of exoglucanases, endoglucanases, β-glucosidases, and oxidative enzymes to produce fermentable glucose units (Wood and McCrae 1979, Eriksson et al. 1990, Perez et al. 2002). Although theoretically efficient yields are possible with purified cellulases, high production costs, inability to depolymerize crystalline cellulose and end product feedback limitations of enzyme reactions severely limit this technology (Perez et al. 2002, Lin et al. 2006, Froese et al. 2008). This problem could be solved by using low-cost, whole organism fungi to carry out or augment the cellulose hydrolysis step. Naturally-occurring wood-rot fungi called brown rot do not decay lignin but naturally hydrolyze wood cellulose, depolymerizing the long glucose chains during early growth using both enzymatic and non-enzymatic (peroxides, oxalate, etc.) processes (Eriksson et al. 1990, Lee et al. 2008, Martinez et al. 2009). Unlike white rot fungi, brown rot fungi do not regulate cellulose hydrolysis based on substrate feedback (glucose availability) (Eriksson et al. 1990, Lee et al. 2008, Martinez et al. 2009). Thus if brown rot fungi were used as a wood pretreatment, the fungus would continually hydrolyze woody substrates until the process is intentionally halted by sterilization. The depolymerized sugars remaining after brown rot fungal colonization consist of oligosaccharides and monomeric hexoses and pentoses, which are suitable for fermentation into ethanol (Lee et al. 2008). This process should even further increase efficiency and ethanol yield from lignocellulosic biomass when combined with enzymatic or chemical hydrolysis. However, staged whole organism fungal treatments have not yet been studied for biopulping lignocellulosic biomass

intended for ethanol production. We hypothesize that combining lignin selective white rot fungi with brown rot fungi in a two-stage process for biopulping lignocellulosic biomass will result in a high yield of low-cost sugars directly useable for the production of ethanol. The objective of the present study is to investigate a two-stage application of whole-organism fungal biopulping for use on wood chips.

Materials and Methods

Fungal isolates, culture conditions, and wood

Fresh cut *Liriodendron tulipifera* (Tulip Poplar) wood chips were collected from a hardwood lumber mill and stored at 4°C until use. *Ceriporiopsis subvermispora* FP-90031-sp (a lignin-selective white rot fungus), and *Postia placenta* Mad-698-R (a brown rot fungus) were obtained from U.S.D.A. Forest Products Laboratory, Madison, Wisconsin, USA. The fungi were cultured in malt extract agar (MEA) and incubated at 28°C for 7-10 days. A fungal plug from the malt extract agar plate was then placed in 500 ml malt extract liquid medium and incubated at 28°C for 7-10 days.

All treatments were conducted in triplicate. For each, 1 gram (oven dry weight) of wood chips was placed in 20 ml scintillation vials and distilled water was added to increase moisture content to 70%. The loosely capped vials were then steam sterilized for 30 minutes. The malt extract liquid fungal cultures were vigorously shaken for one minute before use and 0.2 ml was used to aseptically inoculate each wood chip treatment vial. The controls were also sterilized and 0.2 ml of media from the malt extract cultures was sterile filtered and added to each sterile control vial. The vials were then incubated for 30 days at 28°C for optimal delignification and depolymerization (Clausen and Kartal 2003, Ferraz et al. 2003). After the 30 day colonization period, the vials designated for

two-stage biopulping treatment were steam sterilized for 30 minutes and then inoculated with the second fungal species, using the same inoculation volume and sterile media control as before. The two-stage treatment vials were then incubated for an additional 30 days at 28°C. The treatment vials were then oven dried 24 hours at 104°C.

Chemical Analysis

Gravimetric determination of the holocellulose and Klason lignin content of the sterile controls and decayed samples were performed using previously described microanalytical techniques(Yokoyama et al. 2002, Yeh et al. 2004, Sluiter et al. 2008).

Holocellulose was prepared from extractive free wood meal. For each sample, 100 mg of oven dry weight wood was placed in 20ml round bottom flask. 4 mL of deionized water, 200 mg of 80% sodium chloride, and 0.8 mL of glacial acetic acid was added to each flask and capped. The flasks were then submerged in a 90°C water bath for 1 h. The flasks were cooled and filtered using a sintered glass filter (medium pore size). The holocellulose was washed with deionized water and dried at 104°C before weighing. The holocellulose contents were recorded as a percentage of sample mass.

Klason lignin was prepared from extractive free wood meal. For each sample, 300 mg of oven dry weight wood and 3 mL of 72% sulfuric acid was placed in a 90 mL pressure tube. The sample was then stirred with a glass rod every 15 min for 60 min. The acid was then diluted to 4% concentration by addition of 84 mL of deionized water. The pressure tubes were then capped and autoclaved for one hour at 121°C,cooled overnight at 4°C, and filtered using a sintered glass filter (medium pore size). Acid insoluble lignin was washed with deionized water and dried at 104°C before weighing. Klason lignin contents were recorded as a percentage of sample mass.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed on ground material (40 mesh particle size) from triplicate treatments and controls using a modified method outlined in Shi et al. 2009, including a 1:1.75 mixture of cellulase (22 FPU/g of substrate) (Celluclast 1.5L, Sigma Co.) and β-glucosidase (Novozyme 188, Sigma Co.) and a 3% biomass loading volume (3 replicate samples and 3 replicate controls without enzyme per biopulping treatment). Samples were shaken at 50°C for 72 hours then centrifuged for 10 minutes at 3000 rpm. Aliquots of supernatants were filtered a 0.22 μm filter. Samples were analyzed by HPLC using electrochemical detection and a Carbo-Pac10 carbohydrate column (Dionex) (Lee et. al 2008). The mobile phase was 3 mM NaOH, which was circulated with a flow rate of 0.2 mL/min. Quantification and identification of peaks were performed using dilutions of arabinose, galactose, glucose, xylose, and mannose stock standards. All peaks eluted within 45 min. Glucose contents were normalized to controls without enzymes.

Statistical Analysis

To test the effect of fungal treatments on wood physical and chemical properties, all measured values were analyzed using One-way ANOVA ($\alpha=0.05$) in SAS® 9.2. Duncan multiple range tests among treatments to determine significant differences, were employed. Results were considered significant at $p < 0.05$.

Results and Discussion

Mass loss and degradation in two-stage biopulping

Both white rot (*Ceriporiopsis subvermispora*) and brown rot (*Postia placenta*) fungi were successfully grown on previously colonized biomass by each decay fungus (Figure 1.1). Treatments differed significantly in mass loss ($p=0.0033$) (Figure 1.2). The reverse *P.placenta/C.subvermispora* treatment did not significantly increase the mass loss percentage (Figure 1.2). Steam sterilization of the wood chips induced mass loss in the sterile controls (Figure 1.2). Thus the majority of mass loss amongst the treatments was due to heat solubilization of wood components.

The holocellulose content of the treatments were not significantly different suggesting the fungi did not degrade detectable amounts of wood carbohydrates (Figure 1.3). Both the single stage *P. placenta* and two-stage *P. placenta/C. subvermispora* treatments removed the most acid insoluble lignin, (17-45% of total lignin). Lignin removal was slightly higher in the two-stage treatment *P. placenta/C. subvermispora* (Figure 1.4). The lack of significant difference between the single and two-stage fungal treatments suggests that there was not a stimulative or inhibitory effect caused by the preceding fungal treatment such as previously observed in co-cultures (Chi et al. 2007). The lignin selective white rot fungus *C. subvermispora* did not exhibit normal degradation in the tulip poplar chips, whereas significant lignin removal and little carbohydrate loss were expected (Figures 1.3 and 1.4). Alternatively, the high acid soluble lignin value observed could reflect the highly varied lignin contents of the wood mill chips obtained and may not be considered representative of the known lignin-

selective degradation ability of the fungus (Ericksson et al. 1990, Perez et al. 2002, Choi et al. 2006).

Yield of enzymatic biomass conversion

Two-stage fungal biopulping pretreatments significantly increased the yield of enzymatic hydrolysis in *L. tulipifera* wood chips (Figure 1.5). Based on analysis of soluble glucose, wood that was two-stage biopulped with *C. subvermispora*/*P. placenta* exhibited significant increase in glucose conversion when compared to sterile wood. Comparison with single stage *C. subvermispora* fungal biopulping in previous research suggests that the depolymerization step of *P. placenta* increased glucose solubility (Eriksson et al. 1990, Lee et al. 2008, Shi et al. 2009) (Figure 1.5). The highest levels of soluble glucose were produced using a two-stage treatment of lignin selective white rot fungus (*C. subvermispora*) followed by the brown rot fungus (*P. placenta*) (biopulped wood exhibited a 94% increase in glucose conversion over sterile biomass) (Figure 1.5). The increase in soluble glucose suggests that although there is substantial growth of the fungi and depolymerization of lignin and cellulose, there is not a significant oxidation of the biomass carbohydrates that would reduce theoretical ethanol yield. It is important to note that the two-stage *P. placenta/C. subvermispora* exhibited lower lignin contents but this did not directly translate to increased glucose solubility (Figure 1.4 and Figure 1.5). No significant differences were found in the enzymatic hydrolysis control samples without cellulase treatment, suggesting fungal treatments alone did not significantly increase the fraction of soluble glucose monomers. Cellulose crystallinity and lignin polymerization must be characterized in the two-stage treatments to elucidate the difference in glucose solubility.

Evaluation of two-stage biopulping

Although use of whole-organism fungi as opposed to isolated and purified fungal enzymes might appear to be a step backwards in technology, it is worth pointing out that use of whole organism fungi (i.e. yeasts) is the standard practice and regarded as the mature technology for fermenting sugars into ethanol (Hahn-Hägerdal et al. 2006). Recent fungal pretreatment research has focused on using single stage lignin selective fungal degradation as a method to delignify substrates resulting in some improvements in ethanol yield (Sherestha et al. 2008, Shi et al. 2009, Bak et al. 2009). While advances in downstream processing have been made due to lignin reduction, cellulose recalcitrance remains a significant hurtle to overcome in pretreatment technology. Historically, brown rot fungi were not used as a biopulping pretreatment in paper production due to the rapid degradation of cellulose fibers and the technical advantages of fungal cellulose depolymerization were not explored (Eriksson et al. 1990). However, whole organism brown rot fungi and filtrates have recently been examined as a biological pretreatment in order to depolymerize cellulose and increase enzymatic hydrolysis efficiency in corn fibers and wood chips with significant improvements in downstream ethanol yield (Lee et al. 2008, Sherestha et al. 2010, Fissore et al. 2010). To our knowledge, this is the first work to combine staged whole organism white rot and brown rot fungi together for use as lignocellulosic pretreatment.

The additional feedstock management, sterilization, and prolonged staging time are potential disadvantages of this two-stage process. However, the increased yield of the biopulping process may offset these limitations. Current methods for enzymatic hydrolysis utilize relatively expensive purified cellulase enzymes that are applied to the

biomass in liquid-phase batches (Hahn-Hägerdal et al. 2006, Froese et al. 2008). Considerable fungal biofuel research is based on the isolation and characterization of less expensive fungal enzymes (Hahn-Hägerdal et al. 2006, Lee et al. 2008). However, substantial efforts have not been able to provide industry with low-cost, high-yield cellulase enzymes, primarily due to the high costs of enzyme purification, and feedback limitations inherent in the use of purified enzyme reactions (accumulation of glucose inhibits the rate of hydrolysis) (Eriksson et al. 1990, Hahn-Hägerdal et al. 2006). An industrial process model based on pilot scale testing could determine if two-stage biopulping is an economically viable option for supplementing enzymatic hydrolysis (reducing cost thru yield increase). *Liriodendron tulipifera* wood chips were used in this research due to the abundant availability in the Southeast and limited decay resistance. Additional wood types such as oak and pine will require testing to substantiate widespread use of this two-stage biopulping method for ethanol. The chemical composition of the biopulped material, the effect of biomass type, and the relationship between the two fungal species in single stage co-culture are currently under investigation.

Conclusions

We have demonstrated that specific naturally occurring wood decay fungi can be utilized in an innovative two-stage whole organism approach to significantly increase enzymatic hydrolysis yield of soluble glucose. The potential to augment enzymatic hydrolysis is most promising for continued study. Considering the numerous cost and sustainability advantages over current methods due to reduced enzyme load and increased enzymatic hydrolysis yield, two-stage biopulping technologies could make a significant

contribution to the development of environmentally friendly and sustainable commercial lignocellulosic ethanol production.

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Figure 1.1: *Liriodendron tulipifera* wood chips (≈ 3 x 3 x 0.5 cm) colonized with *Ceriporiopsis subvermispora* seen as a white cottony growth on the surface of the wood.

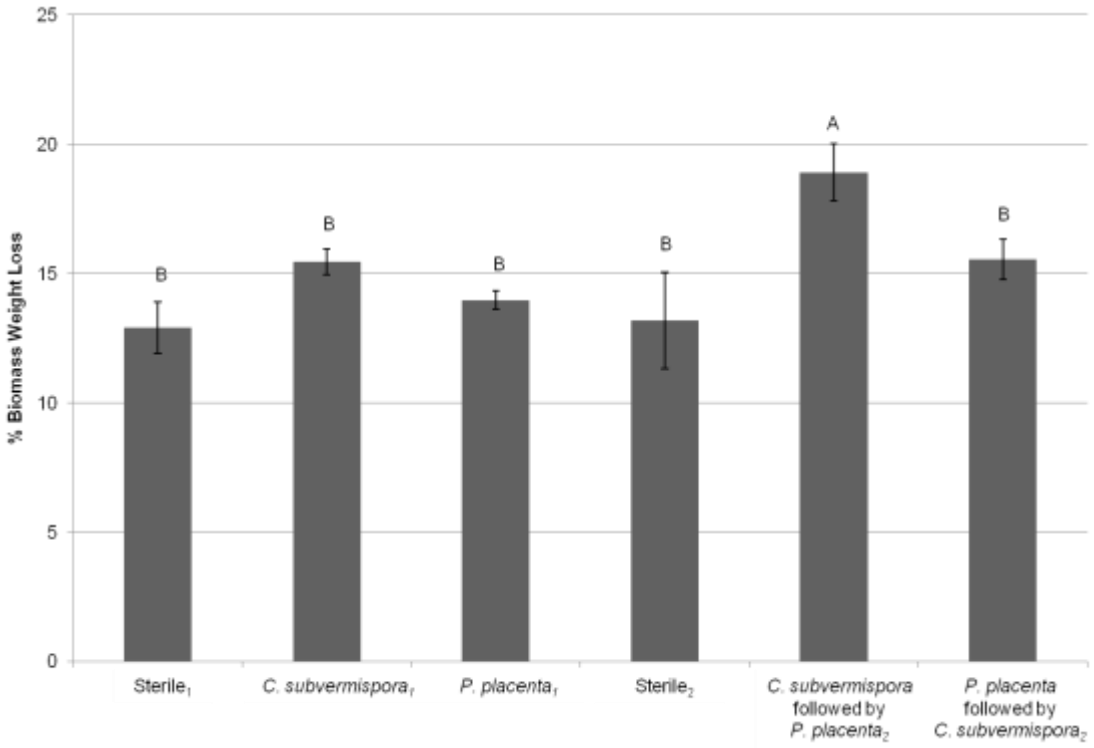

Figure 1.2: Mass loss of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. The number of sterilizations is indicated by the treatment subscript. Fungal treatments n=3, sterile controls n=6. Error bars=1SE.

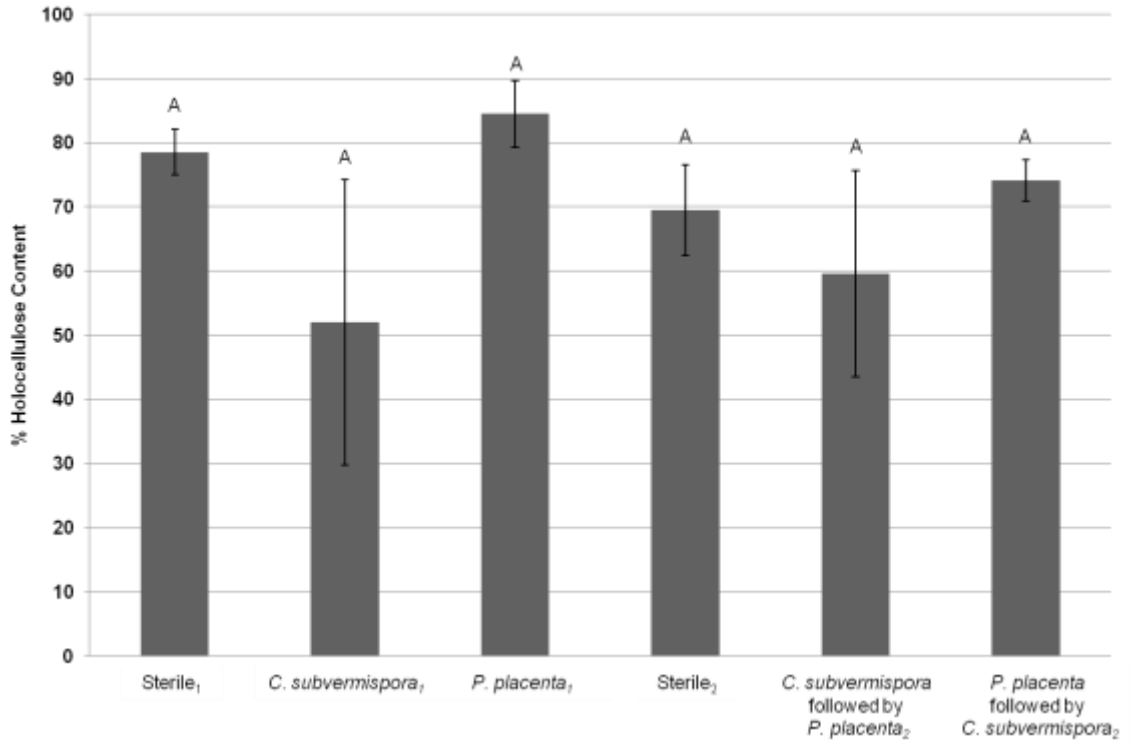

Figure 1.3. Percent holocellulose content of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. Fungal treatments n=3, sterile controls n=6. Error bars=1SE.

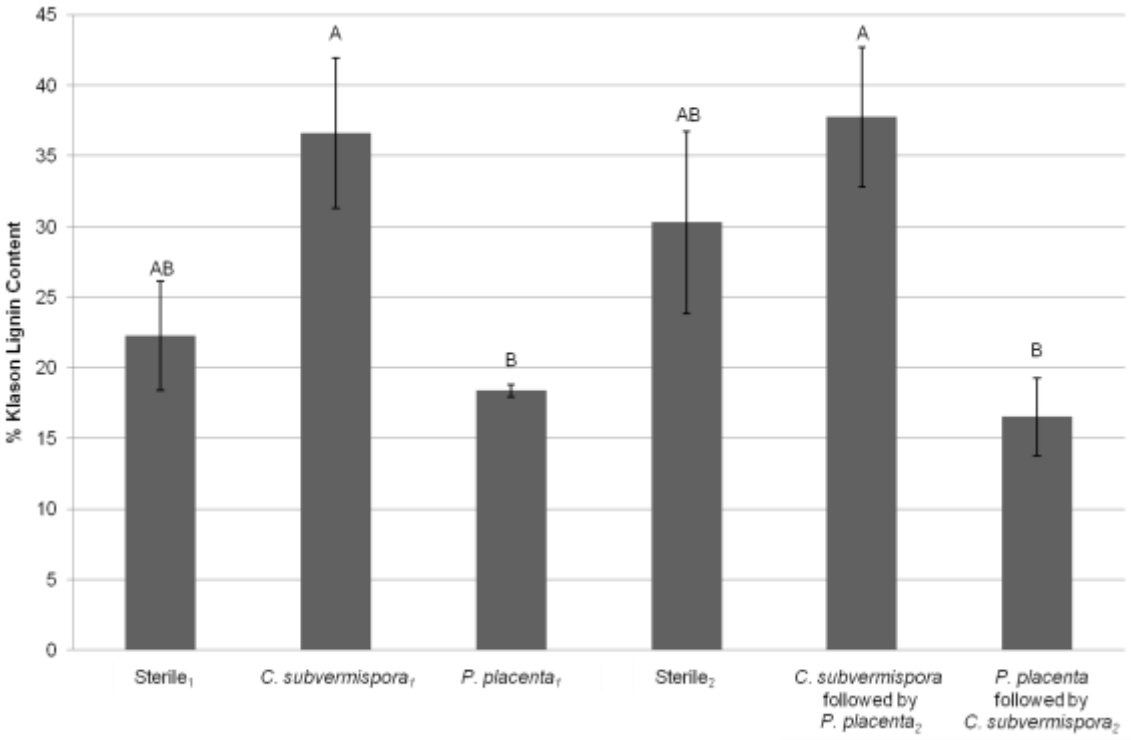

Figure 1.4. Percent Klason lignin content of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. The number of sterilizations is indicated by the treatment subscript. Fungal treatments n=3, sterile controls n=6. Error bars=1SE.

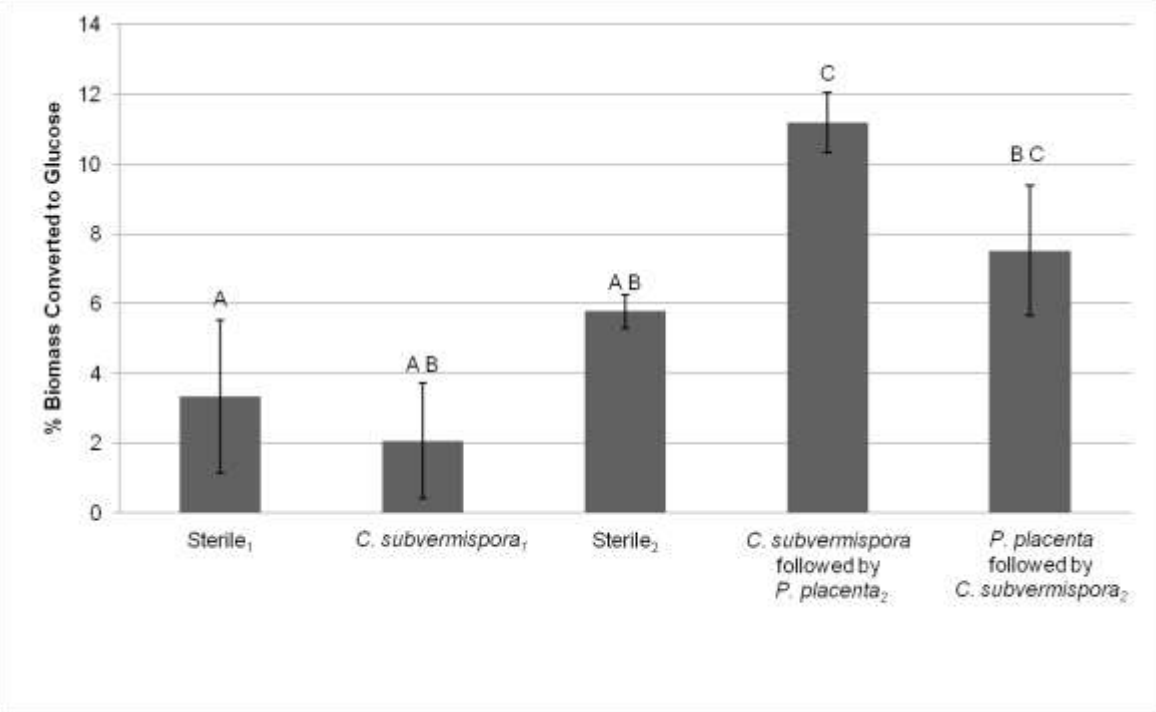

Figure 1.5: Percent of treated *Liriodendron tulipifera* converted to soluble monomeric glucose during enzymatic hydrolysis. Means with the same letter were not significantly different. The number of sterilizations is indicated by the treatment subscript. All treatments n=3. Error bars=1SE.

CHAPTER 2: FUNGAL GROWTH NECESSARY BUT NOT SUFFICIENT FOR EFFECTIVE BIOPULPING OF WOOD FOR LIGNOCELLULOSIC ETHANOL APPLICATIONS

The following chapter was published in the scientific journal *International Biodeterioration and Biodegradation* in 2012. Giles, R.L.; Zackeru, J.C.; Elliott, G.D.; Parrow, M.W. Fungal growth necessary but not sufficient for effective biopulping of wood for lignocellulosic ethanol applications. International Biodeterioration and Biodegradation. 2012, 67, 1-7.

Abstract

A lignocellulosic biomass treatment method utilizing wood rot fungi to improve cellulosic sugar solubilization for ethanol production was modified to provide larger samples for chemical analyses. *Liriodendron tulipifera* wood chips were treated in a novel application of aerobic polypropylene "spawn bags" as experimental bioreactors for biopulping. Treatments were inoculated with culture suspensions of white rot (*Ceriporiopsis subvermispora*) and brown rot (*Postia placenta*) fungi in 40 day incubations. Fungal growth occurred in all treatments, with extensive hyphal coverage of the biomass (ca. >80% of wood chip surfaces). After treatment, physical biodegradation, holocellulose, α-cellulose, and lignin contents of the fungal-biopulped wood chips were comparable to previous results. However, unlike in previous studies, the biopulped wood did not exhibit significant increases in soluble sugars after enzymatic hydrolysis.

These results demonstrate that extensive fungal colonization of biomass is not necessarily sufficient for effective biopulping to increase production of free soluble sugars from wood. Environmental controls on hydrolytic activity by wood rot fungi are poorly known, but we hypothesize that treatment inoculation using nitrogen rich medium may have inhibited lignin-specific fungal hydrolysis of the wood. Future development of effective, standardized biopulping techniques for improving soluble sugar production from lignocellulosic biomass will require optimization of methods including characterization of inoculation nutrients and container effects on fungal metabolic activity.
Keywords: biopulping; brown rot fungi; lignocellulosic ethanol; spawn bags; white rot fungi; wood decay

Introduction

Lignocellulosic biomass such as wood has the potential to replace corn and other seed starches as an abundant, sustainable supply of fermentable sugars for fuel ethanol (Perlack et al. 2005; Lin et al. 2006). However, the abundant sugars contained in wood occur in a complex polymerization of lignin and crystalline cellulose that is difficult to hydrolyze into soluble sugars needed for fermentation (Hahn-Hägerdal et al. 2006). Considerable research has focused on various methods of pretreating wood and other lignocellulosic biomass to improve sugar yield, including mechanical grinding, steam explosion, and acid/base or enzymatic hydrolysis (Sun and Cheng 2002). These methods generally require high-energy input or hazardous toxic chemicals, the associated costs of which currently impede commercialization of lignocellulosic ethanol (Froese et al. 2008).

Wood decay fungi that naturally biodegrade lignocellulosic polymers could potentially be used as a less energy intensive method of biomass treatment for ethanol

production. The industrial process of using whole-organism filamentous wood decay fungi to break down wood or other lignocellulosic biomass is called “biopulping” and has already been used in the paper industry (Akhtar et al. 1992; Young and Akhtar 1997). Filamentous wood decay fungi are generically grouped as either white rot or brown rot fungi, based on differences in their effect on the chemical composition of wood. Each type has distinct depolymerization mechanisms that chemically and mechanically degrade lignin and/or depolymerize cellulose. Lignin selective white rot fungi are capable of enzymatically depolymerizing lignin while simultaneously exhibiting a low uptake of cellulose derived sugars (Otjen et al. 1985; Eriksson et al. 1990; Martinez et al. 2005; Schmidt 2006). Brown rot fungi on the other hand generally do not break down lignin, but rather depolymerize anamorphous and semi-crystalline cellulose by secreting peroxides that cleave long sugar polymers into shorter chains or monomers, which also has the effect of increasing wood fiber permeability (Filley et al. 2002; Kleman-Leyer et al. 1992; Irbe et al. 2006; Howell et al. 2009). Lignocellulosic biomass treatments using a single lignin selective white rot or a single brown rot fungus have previously been examined for their ability to increase downstream ethanol yield (Lee et al. 2008; Shi et al. 2009; Rasmussen et al. 2010; Giles et al. 2011b). In addition, consecutive treatments using both white rot and brown rot fungi to successively delignify and then hydrolyze wood have also recently been shown to significantly increase soluble glucose after enzymatic hydrolysis (Giles et al. 2011a).

Most fungal biopulping research to date has used relatively small amounts of lignocellulosic biomass (<5 grams) in laboratory-scale treatments conducted in small batch “bioreactor” containers such as test tubes, screw-capped culture bottles, or Petri

dishes (Otjen and Blanchette 1985; ASTM 1996; Chi et al. 2007; Giles 2008). The relatively small amounts of biomass treated in such experiments permits only limited chemical analysis of critical wood decay parameters such as lignin, cellulose, and soluble sugar composition, which are required to determine the suitability of biopulping for lignocellulosic ethanol production or other processes. Future comparative studies using different biomass types and/or novel fungal species would benefit from a standardized method for examining larger amounts of biomass to allow for repeated destructive testing of chemical composition during the biopulping process. Furthermore, previous research suggests fungal biopulping is affected by treatment parameters such as biomass quantity, homogeneity, gas exchange, moisture content, and temperature (Akhtar 1997; Young and Akhtar 1997). In the laboratory, these parameters are influenced by treatment container effects, which have varied considerably in previous research. Future comparative studies would benefit from demonstration of a more standardized laboratory bioreactor design that is large enough to provide sufficient biomass for repeated chemical testing, while at the same time supporting active fungal growth, mimicking theoretical industrial scale-up conditions as closely as is experimentally feasible, and remaining relatively inexpensive and easy to use. A goal of this study was to test the suitability of polypropylene "spawn bags" used in commercial mushroom cultivation as bioreactor containers for laboratory-scale fungal biopulping of wood as a treatment process for increasing soluble sugar yield for ethanol production. These bags are inexpensive, hold sufficient biomass to allow repeated chemical testing (>500 g), and have aerobic gas/moisture exchange and contaminant control characteristics suitable for aseptic growth of a variety of fungal species (Upadhyay and Singh 2010). Our hypothesis was that this simple bioreactor

design would yield biopulping efficiency at or better than previous studies using similar biomass and fungal species, while providing larger quantities of biomass for chemical testing compared to previous smaller treatments. The overall objective was to develop and demonstrate a useful, more standardized method for future comparative biopulping studies.

Materials and methods

Wood, fungal isolates, and culture conditions

Fresh cut *Liriodendron tulipifera* (Tulip Poplar) wood chips (~ 3 x 3 x 0.5 cm) were collected from a hardwood lumber mill and stored at 4°C until use. The wood was a mixture of earlywood and latewood chipped from duramen without orientation. The chips were visually inspected to remove bark pieces or defect wood and to verify the homogeneity of all samples before treatment. *Ceriporiopsis subvermispora* FP-90031-sp (a lignin-selective white rot fungus) and *Postia placenta* Mad-698-R (a brown rot fungus) were obtained from USDA Forest Products Laboratory (Madison, WI, USA). The fungi were initially cultured on autoclave-sterilized malt extract agar medium (20 g malt extract L^{-1} in deionized water with 1.5% agar) and incubated at 28°C for 20 days. Fungi were then transferred into autoclave-sterilized modified malt extract liquid medium (20 g malt extract, 1.0 g yeast extract L^{-1} in deionized water) and incubated at 28°C for 20 days prior to use.

All treatments were conducted in triplicate. For each, approximately 100 g (oven dry weight) of wood chips were placed in commercial spawn bags (model FDB1000, Field and Forest Products, Peshtigo, WI, USA). These autoclavable gusseted polypropylene bags are transparent and are equipped with a 10 cm^2 fiber gas exchange

filter. Distilled water was added to increase wood moisture content to 70% for optimal fungal growth (Young and Akhtar 1997). The bags were then steam sterilized at 121°C for 1 hour, allowed to cool, and aseptically inoculated with 10 mL of well-mixed liquid culture suspension of either *C. subvermispora* or *P. placenta*. The bags were then incubated in darkness for 40 days at 28°C, considered optimal for delignification and depolymerization (Clausen and Kartal 2003; Ferraz et al. 2003; Giles et al. 2011a), then steam sterilized at 121°C for 1 hour. *C. subvermispora* treatment bags designated for two-stage biopulping were inoculated with 10 mL of liquid culture of the second fungal species, *P. placenta*. These two-stage treatment bags (and controls) were incubated for an additional 40 days at 28°C then steam sterilized at 121°C for 1 hour. It was determined in previous research that a second sterilization cycle does not increase significant differences in chemical composition and enzymatic hydrolysis between sterile controls (Giles et al. 2011a). The optimal order of staged fungal colonization was previously determined to be *C. subvermispora* followed by *P. placenta* (Giles et al. 2011a).

Chemical analyses

Gravimetric determination of wood extractives, holocellulose, alpha cellulose, and Klason lignin content of the sterile controls and decayed samples was performed using previously described microanalytical techniques (Yokoyama et al., 2002; Yeh et al. 2004; Sluiter et al. 2008; Giles et al. 2011a). Briefly, ground wood meal was oven dried at 104°C before weighing, then extracted for 24 hours using a Sohxlet apparatus to wash 95% EtOH over the samples. The extracted material was then oven dried at 104°C before weighing to determine extractive content which was recorded as a percentage of original sample mass.

Holocellulose was prepared from extractive free wood meal. For each sample, 100 mg of oven dry weight wood was placed in 20 ml round bottom flask. 4 mL of deionized water, 200 mg of 80% sodium chloride, and 0.8 mL of glacial acetic acid was added to each flask. The flasks were then capped and submerged in a 90°C water bath for 1 h. The flasks were then cooled and their contents filtered using a sintered glass filter (medium pore size). The holocellulose filtrate was washed with deionized water and dried at 104°C before weighing. Holocellulose contents were recorded as a percentage of sample mass.

α-cellulose was isolated from 50 mg of isolated holocellulose from each treatment. The holocellulose was treated with 4 mL of 17.5% sodium hydroxide for 30 minutes, and the reaction was then diluted with 4 mL of deionized water and incubated for 30 minutes. The resultant α-cellulose was collected by filtration using a sintered glass filter (medium pore size), washed with deionized water, and dried at 104°C before weighing. α-cellulose contents were recorded as a percentage of sample mass.

Klason lignin was prepared from extractive free wood meal. For each sample, 300 mg of wood (oven dry weight) and 3 mL of 72% sulfuric acid was placed in a 90 mL pressure tube. The sample was stirred with a glass rod every 15 min for 60 min, and then diluted to 4% concentration by addition of 84 mL of deionized water. The pressure tubes were then capped and autoclaved for one hour at 121°C, stored overnight at 4°C, and filtered using a sintered glass filter (medium pore size). The acid insoluble lignin filtrate was then washed with deionized water and dried at 104°C before weighing. Klason lignin contents were recorded as a percentage of sample mass.

Enzymatic hydrolysis

Enzymatic hydrolysis of treatment and control samples was conducted following the methods of Shi et al. (2009) and Giles et al. (2011a) on ground material (40 mesh particle size) from triplicate treatments and controls. A 1:1.75 mixture of cellulase (22 FPU/g of substrate) (Celluclast 1.5L, Sigma Co.) and β-glucosidase (Novozyme 188, Sigma Co.) and a 3% biomass loading volume (3 replicate samples and 3 replicate controls without enzyme per biopulping treatment) was used. Samples were shaken at 50°C for 72 hours and then centrifuged for 10 minutes at 1200 g. Supernatants were filtered (0.22 μm) and stored at 4°C until use.

Determination of sugars

Samples were analyzed by HPLC using a Carbo-Pac10 carbohydrate column (Dionex Corporation, Sunnyvale, CA, USA) and electrochemical detection (Lee et al. 2008). The 3 mM NaOH mobile phase was circulated with a flow rate of 0.2 mL/min. Quantification and identification of peaks were performed using dilutions of arabinose, galactose, glucose, xylose, and mannose stock standards. All peaks were eluted within 45 min. Glucose contents were normalized to controls without enzymes. Samples were also analyzed using the Somogyi-Nelson method for colorimetric determination of reducing sugars (Fournier 2001). Final reducing sugar contents were normalized to controls without enzymes.

Statistical analysis

To test the effect of fungal treatments on wood chemical properties, all measured values were analyzed using One-way ANOVA (α=0.05) in SAS® 9.2. Duncan multiple

range tests were used to determine significant differences between controls and treatments, and among different treatments ($\alpha = 0.05$). Results were considered significant at $p < 0.05$.

Results

Analyses of biopulped wood

Both white rot (*C. subvermispora*) and brown rot (*P. placenta)* fungi grew well in all treatments based on visual inspection. Whereas controls showed no signs of fungal growth or deterioration (Figure 2.1A), treated wood chips exhibited obvious fungal growth and incipient and intermediate fiber decay characteristics consistent with effective biopulping. Mycelial mats and wood fiber degradation were evident even after the chips were dried (Figure 2.1B – 2.1D). Wood chips treated with *C. subvermispora* grew thick mycelial mats with ca. 80% surface coverage on and between wood chips. *P. placenta* growth was less apparent with less observable surface mycelial growth, as was found in previous research (Giles et al. 2011a,b). However, wood chips treated with *P. placenta* were observably darker in color after colonization and exhibited cubical fractures consistent with brown-rot fungal degradation (Figure 2.1C). In all treatments, wood chips were more friable than controls suggesting significant depolymerization and wood fiber strength loss. Wood chips colonized with *C. subvermispora* were easily torn by hand along the longitudinal axis indicating decay typical of white rot fungi (Schmidt 2006). The chips treated with a two-stage colonization exhibited an overall whiter appearance (Figure 2.1D) compared to controls and were easily torn by hand along the longitudinal axis.

Compared to the control and *P. placenta* treatment, the extractives content of the treatments using *C. subvermispora* was significantly higher while the two-stage treatment exhibited the highest extractives content (Figure 2.2). The *P. placenta* treatment extractive content was similar to the control (Figure 2.2). The total bulk proportions holocellulose, α-cellulose, and lignin contents did not exhibit significant differences between the treatments or controls (Figure 2.3, 2.4, and 2.5). The wood from the single-stage and the two-stage fungal treatments exhibited no significant difference in soluble glucose or reducing sugars after enzymatic hydrolysis compared to the control untreated wood (Figure 2.6 and 2.7). An approximate 3.5% conversion of biomass to glucose after enzymatic hydrolysis was observed in all treatments and controls (Figure 2.6). No soluble glucose was detected in enzymatic hydrolysis controls (biomass incubated without cellulase and β-glucosidase).

Discussion

Characterization of biopulped wood

In all treatments, abundant fungal growth, color changes in the wood, and increased wood friability compared to controls indicated the biomass was effectively biopulped in the general sense. The observed lightening of wood color that occurred during white rot colonization suggested lignin was removed from surface fibers (Eriksson et al. 1990; Schmidt 2006), and wood darkening with cubical fractures indicated cellulose degradation typical of brown rot fungi (Schmidt 2006). The overall significant increase in wood friability suggested significant depolymerization and wood fiber strength loss consistent with fungal biopulping (Eriksson et al. 1990; Schmidt 2006). The observed changes in color and chip strength were also consistent with biopulped wood chips and

stems in previous studies (Giles et al. 2011a,b). Overall, the results demonstrate that the polypropylene spawn bag bioreactors examined here supported excellent wood rot fungal growth and general biolpulping of wood under the conditions used here, while providing sufficient biomass for repeated chemical testing. These inexpensive bags are already utilized worldwide in commercial mushroom farming in order to grow many types of related basidiomycete species on woody substrates (Upadhyay and Singh 2010), and the results presented here suggest they could be also be useful in future attempts to standardize laboratory methods for biopulping research.

Extractive content is a measurement of the neutral solvent extractable components of wood, which have negative effects during papermaking (e.g. darkened paper color and reduced adhesive strength). Wood extractives generally consist of fatty acid glycerol esters and phenolic constituents (e.g. lignin monolignols) (Sjöström and Alén 1999). Here we measured extractive content primarily as an indicator of potential soluble sugars that may have been directly produced during the biopulping process. Alternatively, the higher total extractive content measured in the single stage *C. subvermispora* or two-stage successive treatments may be attributable to greater total fungal biomass compared to *P. placenta* only treatments. However, fungal biomass extractive compositions are poorly known and comparison of growth patterns between these two fungal species in *L. tulipifera* have not been previously investigated. Analysis of post-enzymatic hydrolysis samples and controls (containing no cellulase and β-glucosidase) did not exhibit increased monomeric glucose (below), suggesting the increased extractive content of the *C.subvermispora* treated wood was not due to solublized glucose. Further chemical analysis of extractives derived from the two fungal treatments should allow determination

of the effect (if any) of downstream glucose yield or other possible useful metabolic byproducts of degradation.

The total bulk proportions of wood chip holocellulose, α-cellulose, and lignin were not significantly different between any treatments or between treatments and controls. This result was partly expected based on previous research (Filley et al. 2002; Choi et al. 2006; Irbe et al. 2006; Chi et al. 2007; Giles et al. 2011a,b), although selective lignin removal and thus relative holocellulose enrichment was expected in biomass treated with only *C. subvermispora*. Overall, the results demonstrate that bulk proportions of individual wood biomass components (holocellulose, α-cellulose, or lignin) were not significantly altered by any biopulping treatment. It is important to note that the chemical compositions reported are measured percentages of bulk chemical components and not percentage degradation (i.e. wood degradation can occur during biopulping without selective component loss, Giles et al. 2011a,b). These results also demonstrate that although some mass loss typically occurs during biopulping, the percent carbohydrate content of the wood was not significantly reduced. This is important for ethanol applications, since wood carbohydrates are the primary target component for potential downstream fermentation.

Evaluation of the investigated biopulping method for ethanol applications

Biopulping refers to a general process where lignocellulosic biomass is broken down into a less recalcitrant material by selected whole-organism fungi under controlled conditions. Those conditions may likely vary depending upon biomass type and the biopulping goal. In the paper industry, the goal is simply delignification and the separation of wood fibers to produce a lighter-colored, softer material for processing into

paper (Akhtar et al. 1992; Young and Akhtar 1997). However, the goal of biopulping for ethanol is improved access to carbohydrates and/or direct release of soluble fermentable sugars through delignification and cellulose depolymerization (Lee et al. 2008; Shi et al. 2009; Rasmussen et al. 2010; Giles et al. 2011a,b). In previous work, significant increases in soluble glucose were observed using the same fungal isolates in similar staged treatments (Giles et al. 2011a). Unexpectedly, this result was not observed in this study. Although the wood chips in this study were effectively biopulped in the general sense, there was not an increase in soluble sugars after enzymatic hydrolysis compared to controls. This lack of increased soluble sugars in the two-stage treatments may be attributed to the addition of yeast extract in the treatment innoculums, an additive not used in the previous studies (Giles et al. 2011a,b). Fungal cultures used in this study were inoculated onto the wood in a fungal growth medium containing autolyzed yeast extract, a common microbiological supplement with a high organic nitrogen content. This was intended to promote rapid fungal growth and wood chip colonization. However, it may have also altered the specific lignolytic and/or cellulolytic activities of the fungi during biopulping.

In prior research, it has been observed that adding nutrient supplements to wood can significantly inhibit the capability of some lignolytic Polyporales (Aphyllophorales) fungi to degrade wood polymers (Worrall et al. 1997). Specifically, fungal lignin degradation has been found to be significantly greater under nitrogen limitation in some species (Boyle 1998; Eriksson et al. 1990; Schmidt 2006). Low nitrogen availability may be responsible for increasing lignolytic enzyme activity thru ubiquitin and proteosome regulation (Staszczak 2007). The lack of selective lignin removal observed in this study is

supported by previous studies where organic nitrogen (e.g. glutamate) in a liquid inoculum significantly decreased lignin degradation in nitrogen limited wood substrates (Boyle 1998; Choi et al. 2006; Staszczak 2007). Although not specifically known for the species used here, our results suggest that *C. subvermispora* lignin degrading activity may have also been reduced in the present study due to introduction of readily available nitrogen in the inoculums. Reduction of *C. subvermispora*-induced lignin depolymerization would likely decrease subsequent enzymatic hydrolysis efficiency and may have resulted in the reduced soluble sugar production observed in this study. The degree of lignin and cellulose polymerization in wood directly affects the efficiency of enzymatic hydrolysis and therefore the potential soluble sugar yield (Kleman-Leyer et al. 1998; Silverstein et al. 2007; Yoshida et al. 2008). Alternatively, some unknown "container effect" characteristic such as improved gas exchange of the spawn bag bioreactors used in this study may have promoted a type of fungal growth or metabolism poorly suited to the desired biopulping outcome. The fungi grew very well under the conditions imposed here, however it remains possible that vigorous fungal growth may be at odds with specific hydrolytic activity in some species.

Fungal biopulping has great potential as a low-energy, sustainable method for treating lignocellulosic biomass for various applications. By analogy, use of whole-organism fungi (yeasts), as opposed to chemical treatments or purified enzymes, has long been the industry standard for all ethanol production (via fermentation). However, as this study shows, a lack of standardized methods impedes comparative research. The influence of inoculum nutrients requires further characterization with these and other fungal species. Reported methods of inoculation for biopulping vary and include the

application of induced chlamydospores, fungal spores, or nutrient free mycelia suspensions (Leštan and Lamar 1996; Young and Akhtar 1997; Saxena et al. 2001; Bak et al. 2009; Shi et al. 2009; Giles et al. 2011b). Fungal species used in biopulping may not readily produce chlamydospores and only produce spores on basidiocarps, therefore making spore solution inocula impractical. The present study suggests that economical and efficient methods of inoculating wood chips with mycelial suspensions developed for paper biopulping using nitrogen rich media may not be appropriate when the desired result is improved sugar solubilization. Development of biopulping for ethanol (or other) applications requires further investigation of inoculation techniques and suitable bioreactor conditions that promote optimal fungal degradation activity.

Conclusions

We have tested a laboratory scale, simplified batch bioreactor method of fungal biopulping of wood chips for improving the yield of soluble sugars for lignocellulosic ethanol production. The polypropylene spawn bags were highly effective for promoting fungal growth and wood degradation. However, treatment lignin removal and soluble sugar yields were not significantly greater than controls or comparable to previous works. The results suggest that the nitrogen-rich inoculation method used in this study may have inhibited lignin-specific depolymerization by the wood rot fungi used, and highlight a need for further study on the influence of ambient nutrient concentrations and ratios on fungal metabolism and hydrolytic activity during biolpulping. An effective standardized laboratory biopulping method for improved lignocellulosic ethanol production will require further investigation of inoculation methods and potential container effects before commercial biopulping for lignocellulosic ethanol can be realistically adopted.

Acknowledgements

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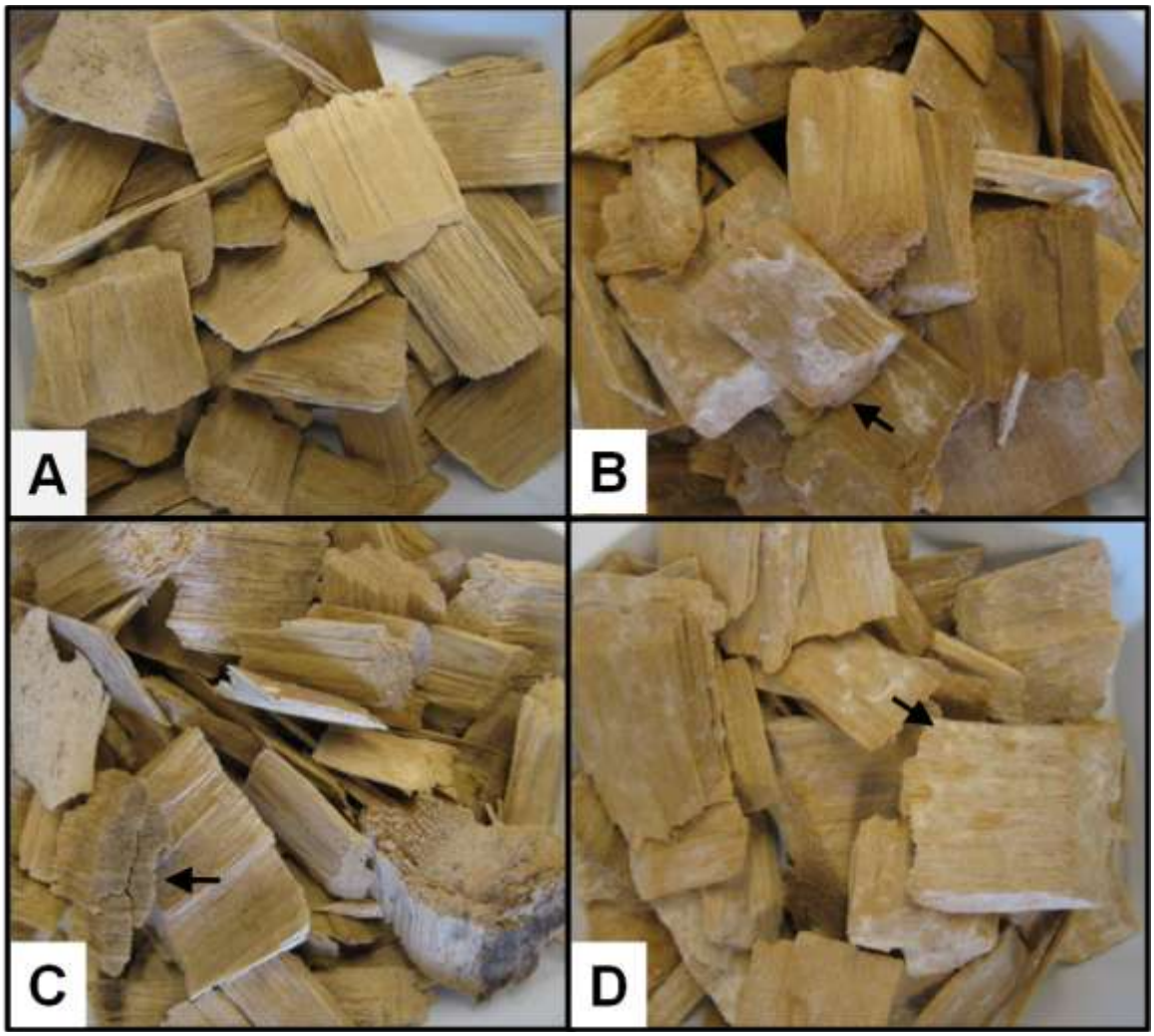

Figure 2.1: Dried *Liriodendron tulipifera* wood chips after biopulping treatment. (A) Control sterile wood chips. (B) Wood chips colonized with *Ceriporiopsis subvermispora*. Arrow indicates chip surface mycelial growth. (C) Wood chips colonized with *Postia placenta*. Arrow indicates cubical fracture typical of brown rot decay. (D) Wood chips colonized with a staged application of *C. subvermispora* followed by *P. placenta*. Arrow indicates chip surface mycelial growth.

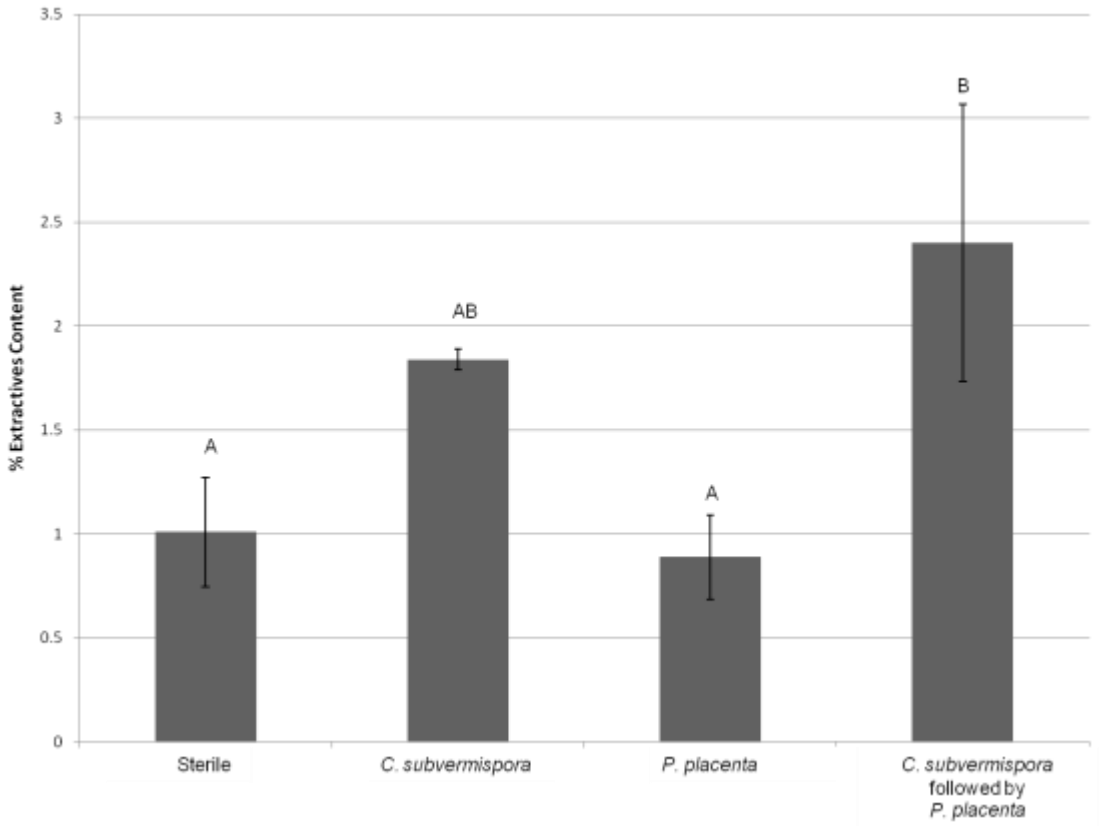

Figure 2.2: Percent extractives content of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. Error bars=1SE.

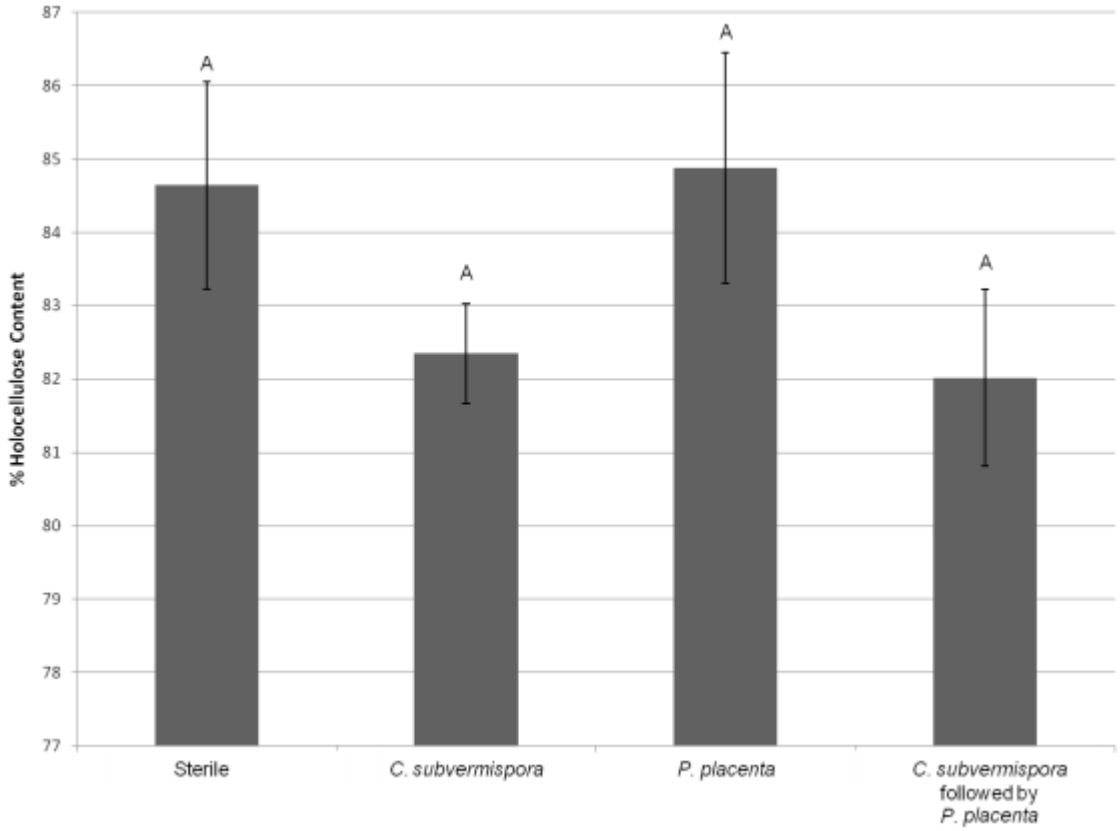

Figure 2.3: Percent holocelullose content of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. Error bars=1SE.

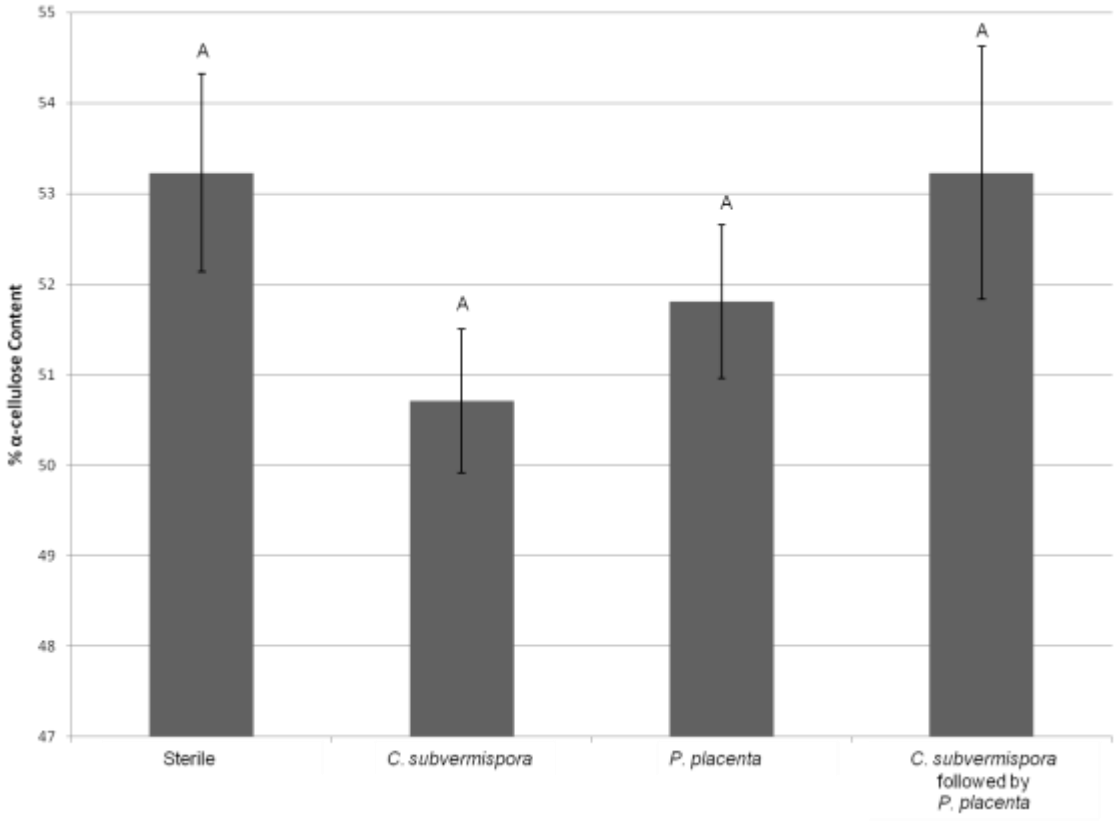

Figure 2.4: Percent α-cellulose content of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. Error bars=1SE.

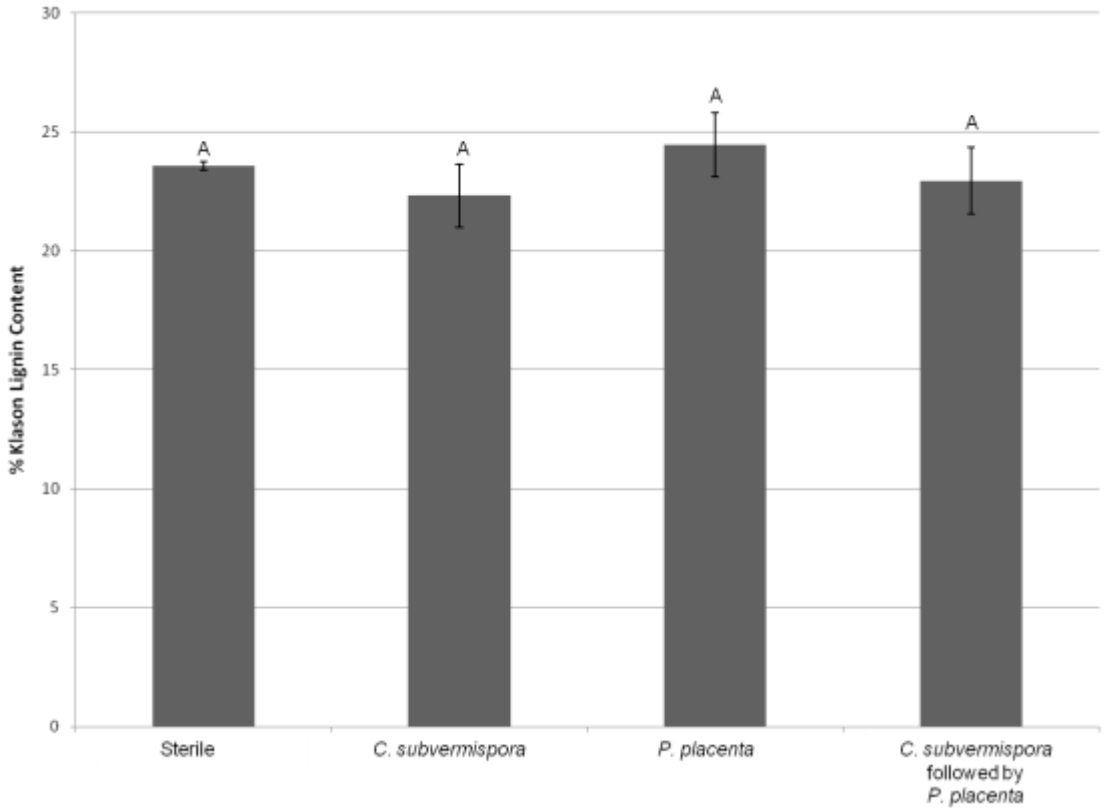

Figure 2.5: Percent Klason lignin content of *Liriodendron tulipifera* wood chips after biopulping. Means with the same letter were not significantly different. Error bars=1SE.

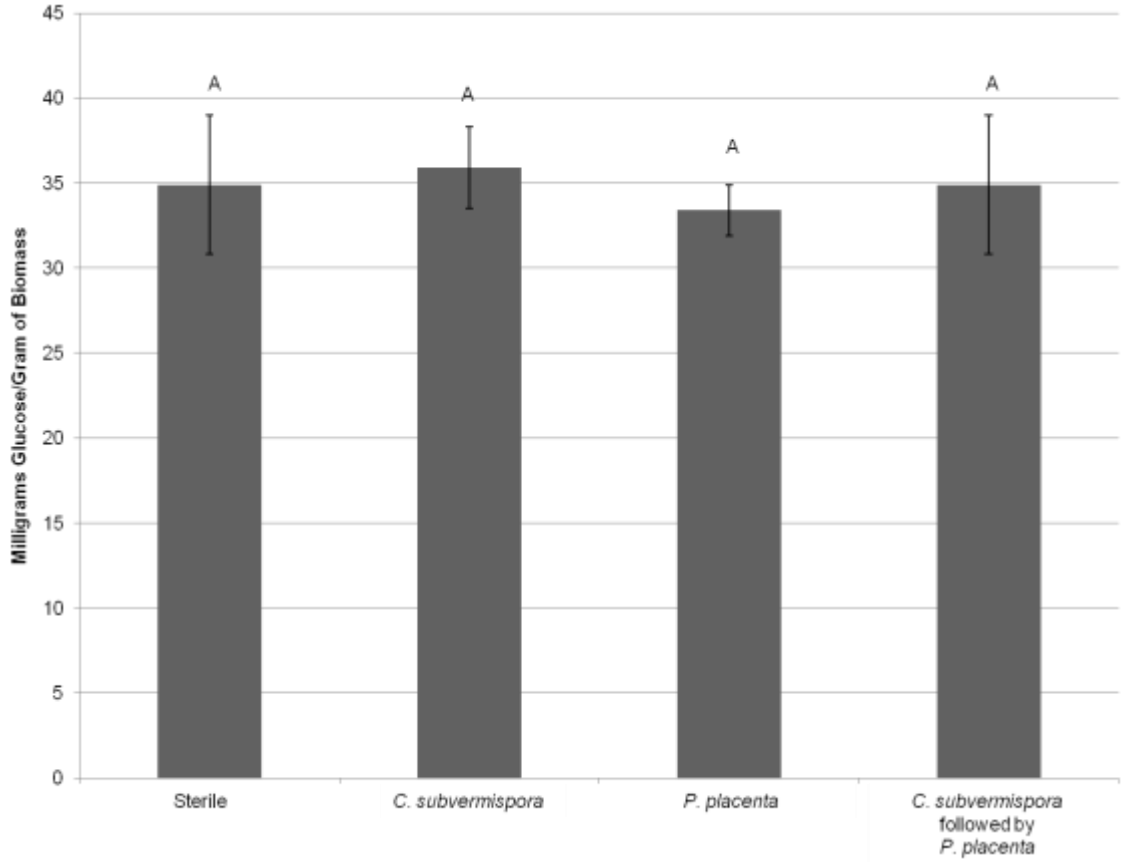

Figure 2.6: Percent of treated *Liriodendron tulipifera* converted to soluble monomeric glucose after enzymatic hydrolysis. Means with the same letter were not significantly different. Error bars=1SE.

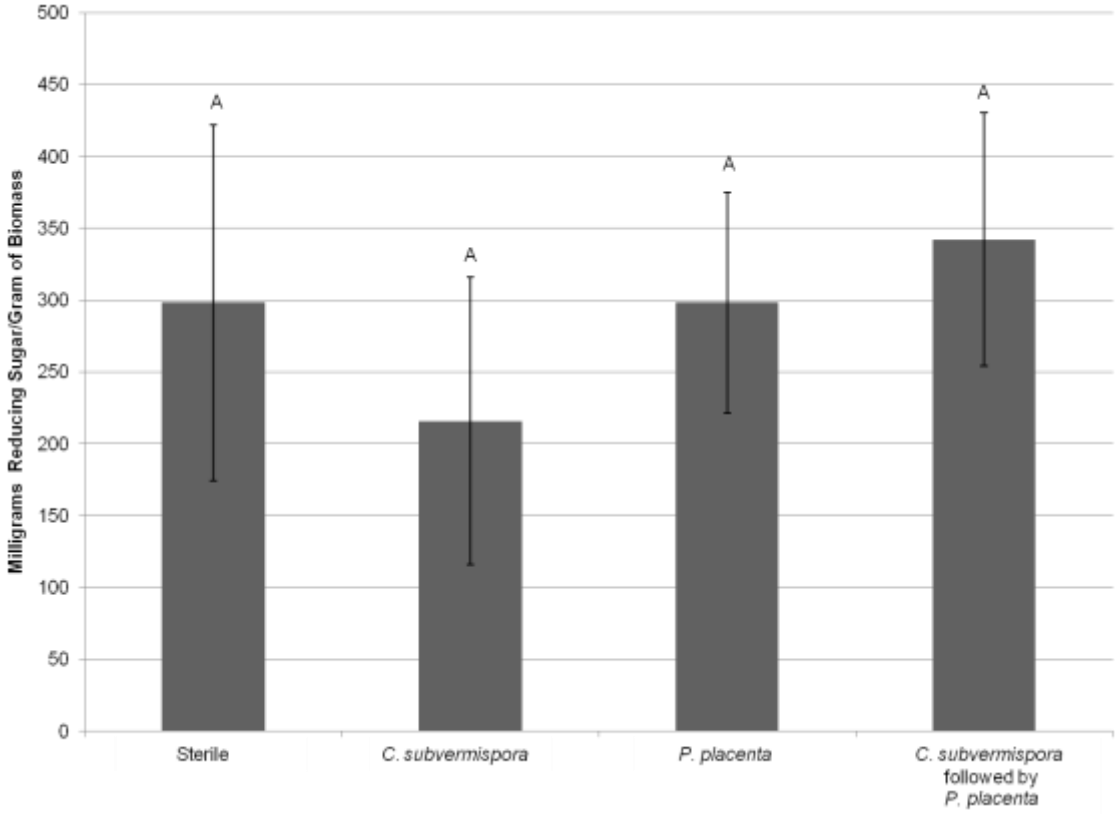

Figure 2.7: Percent of treated *Liriodendron tulipifera* reducing sugars after enzymatic hydrolysis. Means with the same letter were not significantly different. Error bars=1SE.

CHAPTER 3: SINGLE VERSUS SIMULTANEOUS SPECIES TREATMENT OF WOOD WITH *CERIPORIOPSIS SUBVERMISPORA* AND *POSTIA PLACENTA* FOR ETHANOL APPLICATIONS, WITH OBSERVATIONS ON INTERSPECIFIC GROWTH INHIBITION

The following chapter was submitted to the scientific journal *Biomass and Bioenergy* in 2012 and is currently in review. Giles, R.L., Zackeru, J.C., Galloway, E.R., Elliott, G.D., and Parrow, M.W., 2012. Single versus simultaneous species treatment of wood with *Ceriporiopsis subvermispora* and *Postia placenta* for ethanol applications, with observations on interspecific growth inhibition. Biomass and Bioenergy. (Submitted).

Abstract

In order to examine the potential efficacy of simultaneous dual-species fungal treatment of wood for lignocellulosic ethanol production, whole organism fungal biopulping methods utilizing white rot (*Ceriporiopsis subvermispora)* and brown rot (*Postia placenta)* fungi alone or in co-culture were compared for effect on wood microstructure, chemical composition, and enzymatic sugar solubilization. *Liriodendron tulipifera* wood chips exposed for 30 days to *C. subvermispora* and/or *P. placenta* fungi alone or in co-culture exhibited qualitative differences in wood microstructure, but did not significantly differ in final percent composition of holocellulose, α-cellulose, or lignin content compared to controls. All fungal treatments increased the soluble reducing sugar yield of enzymatic hydrolysis by ca. 28-30% over sterile controls. The co-culture fungal

treatment did not significantly differ in reducing sugar yield compared to monoculture treatments, suggesting an unexpected lack of additive or other synergistic species effects on wood degradation using these fungi in co-culture. Paired interaction agar plate assays demonstrated that *C. subvermispora* and *P.placenta* exhibited mutual distance-mediated growth inhibition that was independent of substrate type or availability, suggesting an explanation for the observed lack of degradative synergy between these taxa. This study is the first to report the effects of simultaneous co-treatment with white and brown rot fungi, highlights the need for further optimization of methods to account for specialized fungal degradative mechanisms, and examines the potential influence of competitive interactions in whole-organism biopulping treatments utilizing different taxa.

Keywords: biopulping, wood decay fungi, enzymatic hydrolysis, paper, co-culturing

Introduction

Lignocellulosic biomass potentially represents a significant source of low-cost, renewable carbohydrates for production of biofuels such as ethanol or biobutanol (Hahn-Hägerdal et al. 2006, Dürre 2007). However, the highly polymerized lignin and cellulose in plant cell walls requires intensive pretreatment to release fermentable sugars or other useful substrates for biofuel production, and the current cost of these pretreatments is the primary impediment to commercialization of cellulosic biofuels (Hahn-Hägerdal et al. 2006, Gupta et al. 2009). Recently, searches for low cost methods of biomass pretreatment have sparked interest in natural woody biomass degradation processes. One such process, generally referred to as “biopulping”, utilizes living whole-organism fungi as a low-energy pretreatment to degrade lignocellulosic biomass for industrial paper and/or potential biofuel production (Pérez et al. 2002, Tortella et al. 2008, Vimala and

Nilanjana 2009, Giles et al. 2011, 2012b). Of particular interest are filamentous wood decay fungi belonging to the order Polyporales (within the phylum Basidiomycota), which are ecologically central in the breakdown and nutrient recycling of forest residues (i.e. woody plant lignin and carbohydrate polymers) (Alexopoulos et al. 1996). Forest wood decay fungi of the order Polyporales are grouped polyphyletically as either white rot or brown rot fungi, according to the physical characteristics they impart to wood in the process of degrading it. White rot fungi refers to species that cause a rapid depolymerization of lignin and subsequent exposure of cellulose microfibrils within the wood, giving the substrate a white, cottony appearance (Eriksson et al. 1990, Martinez et al. 2005). Within this group, some taxa are distinguished as "lignin selective white rot fungi" due to their high affinity for the polymer lignin and low cellulose degradation activity. Lignin selective white rot fungi are capable of enzymatically depolymerizing lignin while maintaining a relatively low uptake of cellulose sugars, thus potentially increasing the surface area of embedded cellulose microfibrils within S1, S2, and S3 cell wall layers (Otjen and Blanchette 1985, Eriksson et al. 1990, Martinez et al. 2005, Schmidt 2006). Brown rot fungi are primarily known for degradation of wood in service, such as structural lumber (Martinez et al. 2005). Brown rot fungi tend to selectively degrade cellulose while leaving a modified lignin behind, giving the wood a brown cubical appearance (Eriksson et al. 1990, Martinez et al. 2005). Brown rot fungi rapidly depolymerize anamorphous and semi-crystalline cellulose by secreting peroxides that cleave long carbohydrates into shorter chains, reducing cell wall tensile strength and increasing microfibril permeability (Filley et al. 2002, Kleman-Leyer et al. 1998, Irbe et al. 2006).

In previous research, individual lignin selective white rot or brown rot fungal species have typically been examined for utility as single-species biopulping pretreatments to increase downstream paper fiber or sugar yield from wood or other lignocellulosic biomass (Blanchette 1984, Otjen and Blanchette 1985, Akhtar et al. 1992, Scott et al. 1998, Giles 2008, Shi et al. 2009, Fissore et al. 2010, Rasmussen et al. 2010, Giles et al. 2012a, Wan and Li 2012). Depending upon biomass type and desired breakdown products, exposure to the respective lignolytic and cellulolytic activities of both white rot and brown rot fungi may also be useful as a biopulping strategy. Staged biopulping using successive exposures to white rot and/or brown rot fungi have recently been explored as methods for increasing carbohydrate availability for subsequent enzymatic hydrolysis into glucose (Lee et al. 2008, Giles et al. 2011, 2012b). While staged successive applications of both white and brown rot fungi have been found to significantly increase the sugar yield of enzymatic hydrolysis, the process requirements of staged fungal exposures have disadvantages that may impede commercial adoption. In particular, previous staged treatments required that the biomass be sterilized between fungal exposures, and each fungus required a minimum 30 day colonization period for effective biopulping (Giles et al. 2011). A potential but untested solution to minimize these energy (biomass sterilization) and time (~60 day) costs is simultaneous co-exposure of the biomass to both fungi at the same time. However, it is unknown how the two taxa most commonly used in biopulping experiments (white rot *Ceriporiopsis subvermispora* and brown rot *Postia placenta)* might interact in terms of growth and decay mechanisms when exposed to the same substrate at the same time.

In general, wood decay fungal mycelia interactions are poorly understood but appear to be highly species specific, and range from combative to mutualistic (Rayner and Boddy 1988). To our knowledge, the effects of simultaneous co-culture applications of white and brown rot fungi for biopulping purposes have not been previously examined. Increased wood lignin and holocellulose degradation has been observed in simultaneous co-culture using two different white rot fungi, however brown rot species have not been so examined (Chi et al. 2007, Carabajal et al. 2012). In the present research, we hypothesized that combining the lignin selective white rot fungus *C. subvermispora* with the cellulose-depolymerizing brown rot fungus *P. placenta* in a simultaneous co-culture biopulping treatment would significantly affect wood degradation and chemical composition, compared to respective monospecific fungal treatments of the same duration. The overall goal was to examine the effects of this novel simultaneous fungal treatment on wood biomass in order to explore its potential as a lower-cost, single stage biopulping process. In addition, paired interaction agar plate assays were conducted to examine the gross potential for close growth interactions between *C. subvermispora* and *P.placenta* in a fashion not possible to observe *in situ* within woody biomass.

Materials and Methods

Fungal isolates, culture conditions, and biomass treatments

Ceriporiopsis subvermispora FP-90031-sp (a lignin-selective white rot fungus), and *Postia placenta* Mad-698-R (a brown rot fungus) were obtained from USDA Forest Products Laboratory, Madison, Wisconsin, USA. The fungi were maintained in the laboratory at 28°C in darkness on modified malt extract agar (20 g malt extract, 1.0 g yeast extract, and 20 g agar L^{-1} in deionized water). Fresh cut *Tulip Poplar*

(Liriodendron tulipifera) wood chips were collected from a local hardwood lumber mill and stored at 4°C until use. The wood was a mixture of earlywood and latewood chipped from duramen without orientation. The chips were visually inspected to remove bark pieces or defect wood and to verify the homogeneity of all samples before treatment. Chips were flat in shape and approximately 2-3 cm in diameter.

Single stage biopulping was performed using modification of previously described techniques (Giles et al. 2011, 2012a, 2012b). Prior to inoculation onto wood, *C. subvermispora* and *P. placenta* isolates were subcultured in low nitrogen malt extract agar (20 g malt extract, 20 g agar L^{-1} in deionized water) at 28°C for 7-10 d. A fungal plug from the malt extract agar plate of each species was then placed in 500 ml of low nitrogen malt extract liquid medium (20 g malt extract L^{-1} in deionized water) and incubated at 28°C for 7-10 d. For each treatment, 1 g (oven dry weight) of wood chips was placed into 20 ml glass scintillation vials and distilled water was added to increase moisture content to 70%. The loosely capped vials were then steam sterilized for 30 min. The uninoculated controls were also sterilized. The liquid fungal cultures were vigorously shaken for one min before use and 0.2 ml was used to aseptically inoculate each wood chip monoculture treatment vial. Co-culture treatment vials were inoculated with 0.2 ml of both *C. subvermispora* and *P. placenta.* All treatments and controls were conducted in triplicate. All vials were incubated for 30 d at 28°C for optimal delignification and depolymerization (Clausen and Kartal 2003, Ferraz et al. 2003, Giles et al. 2011). After the 30 d treatment period, all vials (including uninoculated controls) were steam sterilized for 30 min to halt biological activity.

Microscopy

Random wood specimens were selected from each treatment and control for observation of fungal growth and cell wall degradation. Samples were oven dried at 104°C, mounted on aluminum stubs with carbon tape, and coated with 30 nm Au-Pd in a plasma chamber. Samples were imaged with a JEOL JSM-6460 low vacuum scanning electron microscope (SEM) at an accelerating voltage of 5 kV to reduce sample charging.

Chemical analyses of wood

Gravimetric determination of wood holocellulose, α-cellulose, and Klason lignin content of the sterile controls and biopulped samples was performed using previously described microanalytical techniques (Yokoyama et al. 2002, Yeh et al. 2004, Sluiter et al. 2008, Giles et al. 2011). Wood was ground into meal (40 mesh particle size), oven dried at 104°C, weighed, and then extracted using a Sohxlet apparatus to wash 95% EtOH over the samples for 24 h. The extracted material was then oven dried at 104°C and weighed again.

For each treatment, holocellulose was quantified from 100 mg of oven dry weight extractive free wood meal. Each sample was placed into a 20 ml round bottom flask and 4 mL of deionized water, 200 mg of 80% sodium chlorite, and 0.8 mL of glacial acetic acid was added. The flasks were then capped and submerged in a 90°C water bath for 1 h. The flasks were then cooled and their contents filtered using a sintered glass filter (medium pore size). The holocellulose filtrate was washed with deionized water and dried at 104°C before weighing. Holocellulose contents were recorded as a percentage of original sample mass.

α-cellulose was isolated from 50 mg of isolated holocellulose from each treatment. The holocellulose was treated with 4 mL of 17.5% sodium hydroxide for 30 min, and the reaction was then diluted with 4 mL of deionized water and incubated for 30 minutes. The resultant α-cellulose was collected by filtration using a sintered glass filter (medium pore size), washed with deionized water, and dried at 104°C before weighing. α-cellulose contents were recorded as a percentage of original sample mass.

Klason lignin was prepared from extractive free wood meal. For each sample, 300 mg of wood (oven dry weight) and 3 mL of 72% sulfuric acid was placed in a 90 mL pressure tube. The sample was stirred with a glass rod every 15 min for 60 min, and then diluted to 4% concentration by addition of 84 mL of deionized water. The pressure tubes were then capped and autoclaved for one hour at 121°C, stored overnight at 4°C, and filtered using a sintered glass filter (medium pore size). The acid insoluble lignin filtrate was then washed with deionized water and dried at 104°C before weighing. Klason lignin contents were recorded as a percentage of original sample mass.

Enzymatic hydrolysis and determination of sugar yield

Enzymatic hydrolysis of treatment and control samples was conducted following the methods of Shi et al. (2009) and Giles et al. (2011, 2012a) on ground material (40 mesh particle size) from triplicate treatments and controls. A 1:1.75 mixture of cellulase (22 FPU/g of substrate) (Celluclast 1.5L, Sigma Co.) and β-glucosidase (Novozyme 188, Sigma Co.) with a 3% biomass loading volume was used (3 replicate samples and 3 replicate controls without enzyme per biopulping treatment). Samples were shaken at 50°C for 72 h and then centrifuged for 10 min at 1200 g. Supernatants were filtered (0.22 μm pore size filter) and stored at 4°C until use. Samples were analyzed using the

Somogyi-Nelson method for colorimetric determination of reducing sugars (Fournier 2001). Final reducing sugar contents were normalized to controls without enzymes.

Agar plate assays for interspecific growth interactions

Fungal growth interactions were examined by spot-inoculating agar Petri plates on opposite sides (approximately 40mm apart) of the assay plate in a pairwise matrix fashion (e.g. *C. subvermispora* versus *C. subvermispora*, *P. placenta* versus *P. placenta*, or *C. subvermispora* versus *P. placenta*). Square agar plugs ($1cm^2$) cut from stock malt extract agar plate cultures were used to aseptically inoculate each assay plate at opposing sides. To examine the potential effects of substrate type or availability on growth interactions, three different assay media types were used: 1) modified malt extract agar (MEA) (20 g malt extract, 1.0 g yeast extract, and 20 g agar L^{-1} in deionized water); 2) ground wood agar (GWA) (20 g of 40 mesh ground *L. tulipifera* wood, 20 g agar L^{-1} in deionized water; and 3) 2% water agar (WA) (20 g agar L^{-1} in deionized water). All treatment combinations and sterile controls were conducted in triplicate. The assay plates were incubated inverted at 28°C in darkness for 30 d to allow adequate time for the fungal mycelia to spread across the surface and meet in the middle of each plate, and observed for gross interactions at the central convergence zone between the two opposing mycelial growth fronts. Interactions between opposing mycelia were assessed visually using the classification methods described by Rayner and Boddy (1988). Plates were observed again after 60 d to assess longer-term interactions.

Statistical analysis

To test the effect of fungal treatments on wood chemical properties, all measured values were analyzed using One-way ANOVA (α=0.05) in JMP® 9.0.2 (SAS Institute,

Cary, North Carolina, USA). Tukey-Kramer tests were used to determine significant differences between controls and treatments, and among different treatments ($\alpha = 0.05$).

Results and Discussion

Biopulping effects on biomass microstructure and chemical composition

In all treatments, the tested fungi grew as visible mycelial mats that covered the surface of the wood chips within the experimental period, indicating robust colonization of the biomass as previously observed (Giles et al. 2011, 2012b). Deep intrastructural fungal colonization was also evident in all treated samples of ground wood (before enzymatic hydrolysis). However, the *P. placenta*-only treatment exhibited qualitatively more wood cell surface hyphal growth at the macro scale and in SEM analysis than the other two treatments (Figure 3.1), and all treatments with *P. placenta* (both single and dual-species) exhibited qualitatively more abundant hyphal growth on the exterior of the wood chips than the *C. subvermispora*-only treatments. *P. placenta* and *C. subvermispora* hyphae are not readily distinguishable based on gross morphology (Gilbertson and Ryvarden 1986, 1987). Therefore, it was not possible to determine the relative abundance of either species in co-culture treatments, or indeed even if both species grew in co-culture. It remains possible that *P. placenta* significantly outcompeted *C. subvermispora* in co-culture on this biomass type, as may be suggested by relative growth performance in single-species treatments and subsequent chemical composition measurements (below).

Increased friability and fiber separation along the longitudinal axis was observed in the *C. subvermispora* and co-culture treatments suggesting lignin depolymerization and separation along the middle lamella, as expected based on previous research (Ferraz

et al. 2003, Choi et al. 2006, Schmidt 2006, Giles et al. 2012b). Cubical fractures and wood darkening was observed in both *P. placenta* and co-culture treatments, indicative of significant cellulose depolymerization and microfibrial structure loss (Schmidt 2006, Giles et al. 2012b). Observed macro-scale longitudinal separation and cubical fracturing were also evident in SEM analysis of ground wood. Although *C. subvermispora* and *P. placenta* hyphae could not be distinguished from one another in co-culture treatments, an increased fibrillation of the cell wall S1 and S2 layers were observed in the wood treated with a simultaneous co-culture compared to the other treatments (Figure 3.2).

Post-treatment wood chemical compositions were determined as percentages of final bulk chemical composition, not percent component degradation (Table 3.1). The holocellulose, α-cellulose, and lignin contents did not exhibit significant differences between the treatments or controls (Table 3.1). The observed increase in *P. placenta* holocellulose percentage over controls was inversely proportional to the decrease in α-cellulose when compared to sterile control. This suggests that *P. placenta* selectively degraded the α-cellulose within the *L. tulipifera* cell wall, increasing the hemicellulose percentage of the biomass. This incipent decay hemicellulose "enrichment" has been observed in previous studies and is typical of brown rot fungal degradation (Eriksson et al. 1990, Martinez et al. 2005, Martinez et al. 2009, Giles et al. 2011, 2012b). Recently, it has been suggested that brown rot fungi remove portions of hemicelluloses, increasing digestibility and enzymatic hydrolysis (cellulases) yield (although an extended 56-day colonization time was used to evaluate biopulped biomass; Monrroy et al. 2011). Lignin content in the *P. placenta* treatment was not significantly different from the control as expected based on previous research (Filley et al. 2002, Irbe et al. 2006).

The holocellulose contents were significantly lower in the *C. subvermispora* treatment when compared to the *P. placenta* treatment (an approximate 10% difference was detected) (Table 3.1). A similar decrease in carbohydrates was observed in previous studies examining the effect of *C. subvermispora* on hardwoods (*L. tulipifera and Populus tremuloides*) (Giles et al. 2012a, 2012b). *C. subvermispora* lignin depolymerization (demethylation of syringyl alcohol) has been previously demonstrated to occur without significant bulk lignin loss, an effect that contributes to improved fiber digestibility in paper manufacturing (Ahktar et al. 1992, Choi et al. 2006, Ferraz et al. 2008).

Unexpectedly, the holocellulose, α-cellulose, and lignin contents of the co-culture treatment was similar to sterile wood suggesting a reduced degradation capability of the co-culture treatment when compared to single stage applications (Table 3.1). Co-culture applications of different taxa of white rot fungi on wood have been reported to exhibit interactions that stimulate degradation of lignin and lignosulfonates; however, distinctive and variable species and strain effects were observed (Wolfaardt et al. 2004, Chi et al. 2007, Howell et al. 2009). In the present study, the observed lack of difference in chemical composition when compared to the single-species applications of fungi may suggest that one species (e.g. *P. placenta*) may have significantly outcompeted the other in co-culture under the conditions imposed in this study. Alternatively, if both species exhibited significant growth, their respective degradative effects on the tested biomass may have been ameliorated by poorly-understood aspects of interspecific fungal competition (Rayner and Webber 1984, Rayner and Boddy 1988).

Evaluation of co-culturing for lignocellulosic ethanol production

Both single-species and co-culture fungal treatments significantly increased the yield of reducing sugars after enzymatic hydrolysis when compared to the control untreated wood (Figure 3.3). An approximate 8% conversion of biomass to reducing sugars after enzymatic hydrolysis was observed in sterile controls, whereas an approximate 25% conversion of biomass to reducing sugars after enzymatic hydrolysis was observed in all fungal treatments (Figure 3.3). Overall, the fungal treatments significantly increased the enzymatic conversion of holocellulose to reducing sugars from 12% in sterile controls to 36-39%. These results were expected based on previous reports of increased solubility of carbohydrates after fungal pretreatments of woody biomass (Monrroy et al. 2009, Shi et al. 2009, Giles et al. 2011, 2012a).

There was no significant difference in reducing sugar yield between the single species and dual-species co-culture fungal treatments (Figure 3.3). This unexpected observation suggests that there were no inhibitory or additive effects between the two fungi under these conditions. The reasons for this are unclear; it remains possible that only one species grew well in co-culture, or that both taxa grew but competed with one another in some manner that negatively influenced the potential additive effects of their specific degradative mechanisms, as previously discussed. The present study represents only an early step toward understanding the potential for co-culturing these two fungi on wood for bio-ethanol applications. Results may differ depending upon biomass type (i.e. different lignin:holocellulose ratios), or with incubation parameters such as temperature, treatment mass, or gas exchange (Giles et al. 2012b). Optimized application techniques could include incremental application of each isolate, isolate specific nutrient innoculant,

spatial inoculation, and other dynamic incubation parameters. In a biopulping for ethanol application, the biomass could be ground or colonized in a liquid-state bioreactor allowing somatic growth and supplementing extracellular degradation mechanisms via the liquid phase. Bioreactors using immobile cultures of wood decay fungi to detoxify aromatic molecules from wastewater have been successfully designed (Couto 2009). A similar static bed culture system could be modified to simultaneously treat wood slurry with multiple fungi. The unknown effects of particle size and biomass type on co-culture growth must be further characterized in order to develop a single stage co-culture biopulping process for commercial applications.

Evaluation of interspecific growth interactions

As previously discussed, the observed lack of additive degradation effects in the dual-species co-culture treatments suggested the possibility of significant interspecific competition between *C. subvermispora* and *P. placenta* under the conditions of this study. Such interactions would be difficult or impossible to observe *in situ* in woody biomass due to the morphological similarity of these taxa, so agar plate growth assays were conducted to examine the potential for interspecific interaction in a more observable and interpretable format. Both *C. subvermispora* and *P. placenta* fungal strains quickly colonized malt extract agar (MEA), ground wood agar (GWA), and water agar (WA), with dense hyphal growth spreading in all treatments from the two plate margin inoculation points into the plate center within 10 days. In all same-species assays (i.e. *C. subvermispora* versus *C. subvermispora*; *P. placenta* versus *P. placenta*), the opposing mycelial growth fronts simply merged together in the central zone, creating a single confluent mycelial mass across the surface of each plate (Figure 3.4). These results

indicated a lack of intraspecific competition, as expected. In contrast, the opposing mycelial growth fronts in all dual-species assays (i.e. *C. subvermispora* versus *P. placenta)* did not merge together (Figure 3.4). Rather, growth of each taxon halted at a within-treatment uniform distance from one another (ca. 0.4-7.7 mm), with a clear "inhibition" zone of little to no mycelial intrusion separating the two opposing fungal species. This zone of inhibition was clearly established within 30 days and remained constant over an observation period of 60 days with no indication of species dominance or replacement. Growth inhibition was apparently bilateral, with neither fungus exhibiting dominance or replacement growth patterns, suggesting mutual inhibition. Furthermore, the observed growth inhibition did not vary with substrate type, and even occurred on non-nutrient (water only) agar. This suggests that interspecific growth inhibition between these taxa was independent of substrate type or availability. Overall, the observed gridlocked growth pattern with a clear zone of mutual inhibition indicated interspecific growth inhibition at a distance, likely mediated by production/exchange of diffusible molecules (Rayner and Boddy 1988). There was no evident antibiosis at distances greater than 8 mm between the two mycelial mats anytime during incubation, suggesting these two isolates can coexist within the same biomass, but not in close association. Based on agar plate assays, local fine-scale compartmentalization of each fungus may occur in co-cultured biomass, leading to pockets of brown and white rot fungal growth encapsulated within uncolonized wood. Such pockets were not observed on the wood surface or in thin sectioning (Section 3.1), but would be difficult to discern due to the morphological similarity of these two taxa.

The specific mechanisms of inhibition between wood decay Basidiomycetes used in biotechnology applications are poorly understood. However, it is clear that small molecule exchange between Ascomycete fungi illicit species-specific growth inhibition and other physiological responses (Rayner and Webber 1984, Cotier and Muḧlschlegel 2012). Small diffusible molecules (e.g. farnesol and ammonia) can affect growth differentiation and cell proliferation in Ascomycete yeasts (Palkova et al. 1997, Sprague and Winans 2006, Nigam et al. 2011). Quorum sensing has not yet been characterized in Basidiomycetes, although similar mechanisms of molecular communication are expected to occur across fungal taxa (Atkinson and Williams 2009). Growth or other ecological interactions between C. *subvermispora* and *P. placenta* have not been explored previously, but the results of this study demonstrate a pattern of growth inhibition that may impact their utility in co-culture applications. The observations also suggest that although these fungi may inhibit each other's growth, this inhibition operates at close proximity through a diffusion mechanism that would still permit co-colonization at relatively close scales in highly polymerized woody substrates.

Conclusions

A novel dual-species, single-stage fungal biopulping method utilizing white rot and brown rot fungi was examined as a potential pretreatment of wood for lignocellulosic ethanol applications. The effects of co-culturing white rot and brown rot fungi have not been previously reported and these initial results could inform further developments in future multi-species biopulping processes. Co-culture application of white and brown rot fungi did not significantly decrease or increase the holocellulose content of the wood when compared to controls, suggesting the method does not stimulate increased

carbohydrate degradation. The examined co-culture biopulping treatment affected fungal holocellulose decay and ground wood particle morphology when compared to single-species applications of fungi. Additionally, the co-culture treatment did not significantly alter reducing sugar yield when compared to treatments of single applications of each fungus, suggesting no stimulatory (or inhibitory) effect on sugar solubility. Paired interaction agar plate assays demonstrated that the *C. subvermispora* and *P.placenta* strains examined exhibit mutual growth inhibition that is independent of substrate type or availability. This interaction may reduce the utility of co-culture biopulping applications using these taxa, and highlights the need for further study of the effects of fungal interspecific interactions on lignin degradation and cellulose depolymerization in different biomass types and under different incubation conditions.

Acknowledgements

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Table 3.1: Mass loss and chemical composition of *Liriodendron tulipifera* wood chips after degradation, means ± 1SE, n=3.

Treatment	N	Holocellulose % ±SE	α-cellulose % ±SE	Klason Lignin % ±SE
C. subvermispora	3	66.38 ±2.40^{A}	37.04±4.42^{A}	12.66 ±2.13^{A}
P. placenta	3	73.09 ±0.45^{B}	40.65 ±2.71^{A}	15.72 ±2.27^{A}
C. subvermispora + *P. placenta*	3	68.93 ±1.92^{AB}	40.49 ±0.70^{A}	11.07 ±1.30^{A}
Sterile	3	71.06 ±0.34^{AB}	44.29 ±2.34^{A}	11.94 ±2.27^{A}

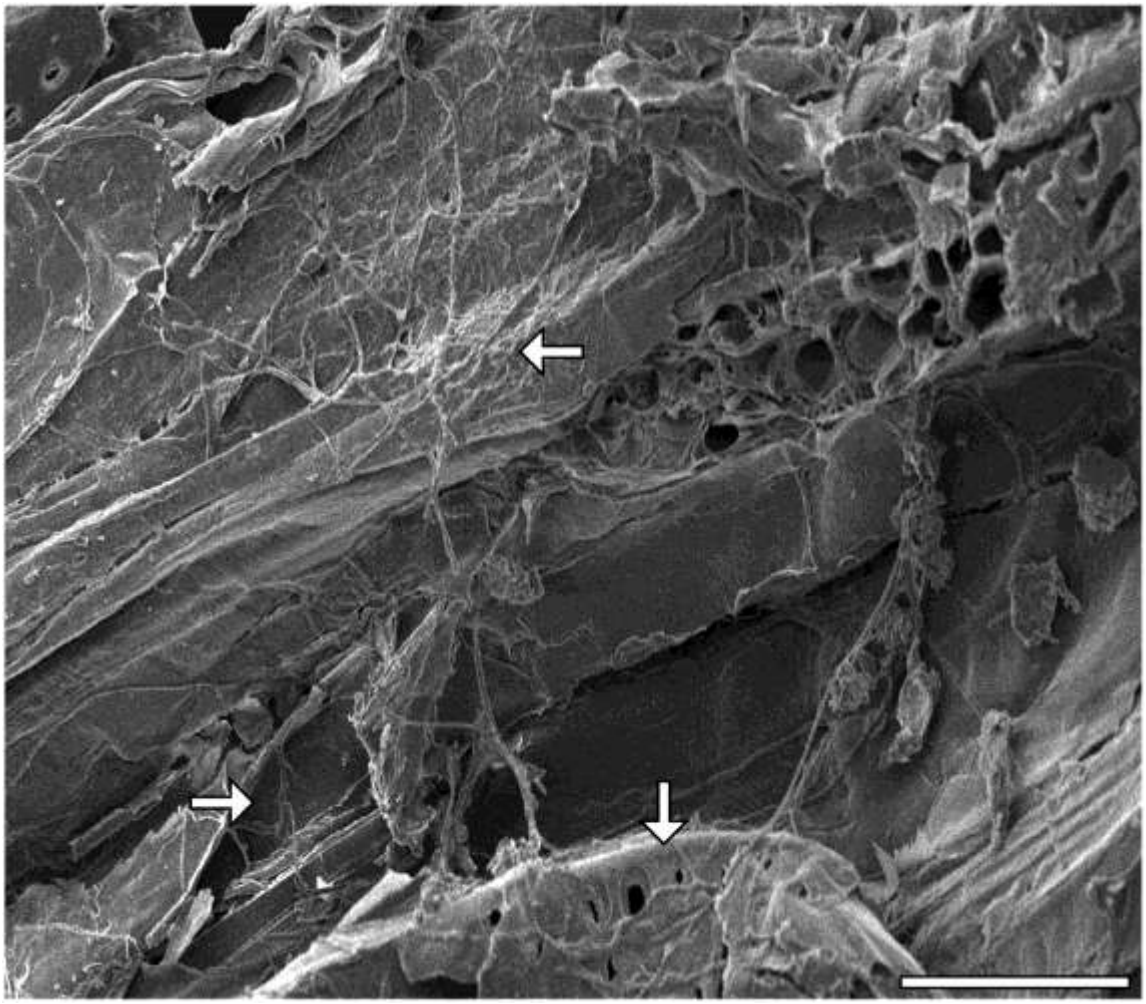

Figure 3.1: *Postia placenta* mycelia (arrows) in dried ground wood. Bar equals 50 m.

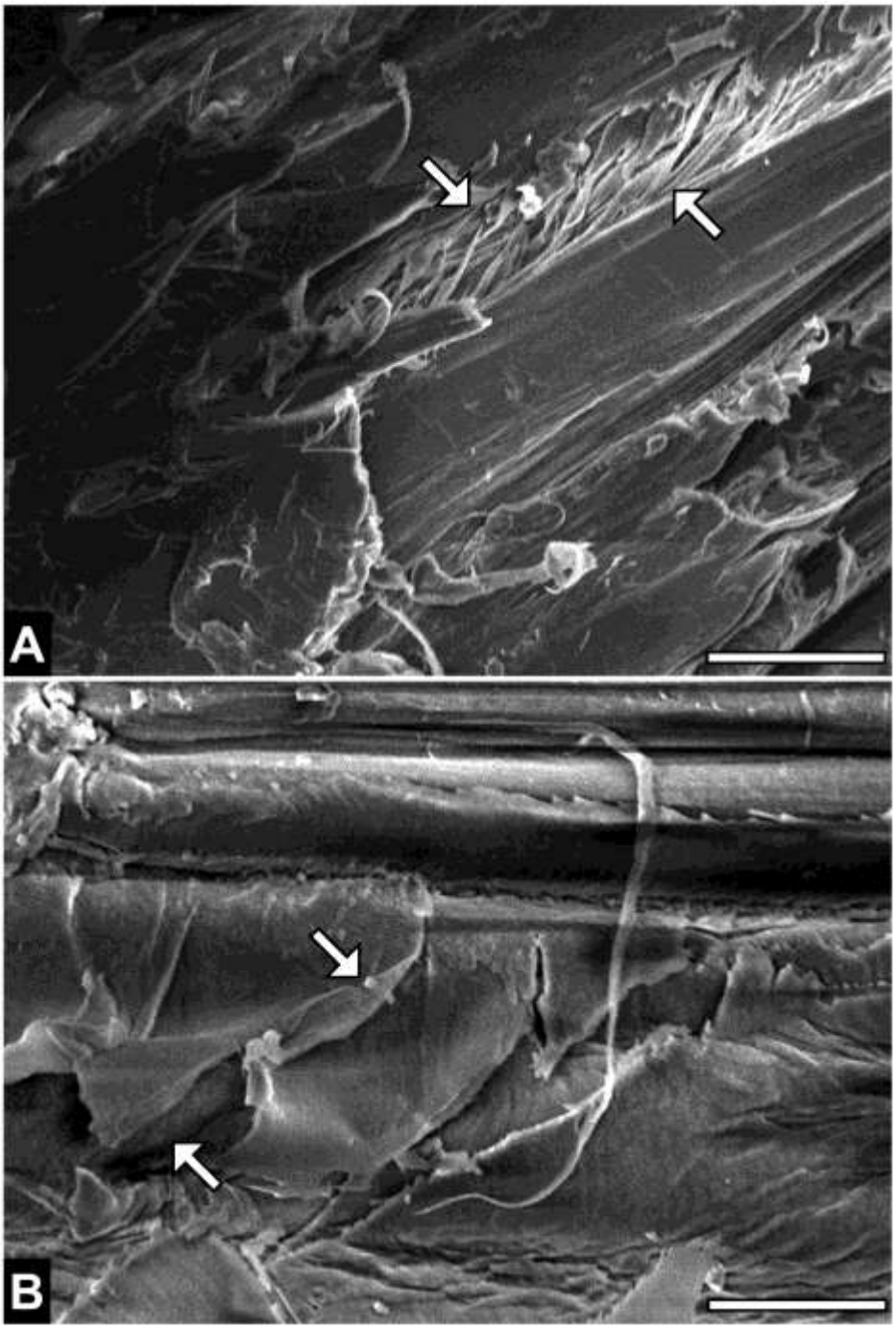

Figure 3.2: Fibrillation of the cell wall layers (arrows) in ground wood treated with a simultaneous co-culture of *Cerioporiopsis subvermispora* and *Postia placenta* for 30 days. A) Ribbon shaped fibrillation of the S2 layer. Bar equals 10 um. B) Flaked fibrillation of S1 layer. Bar equals 5 µm.

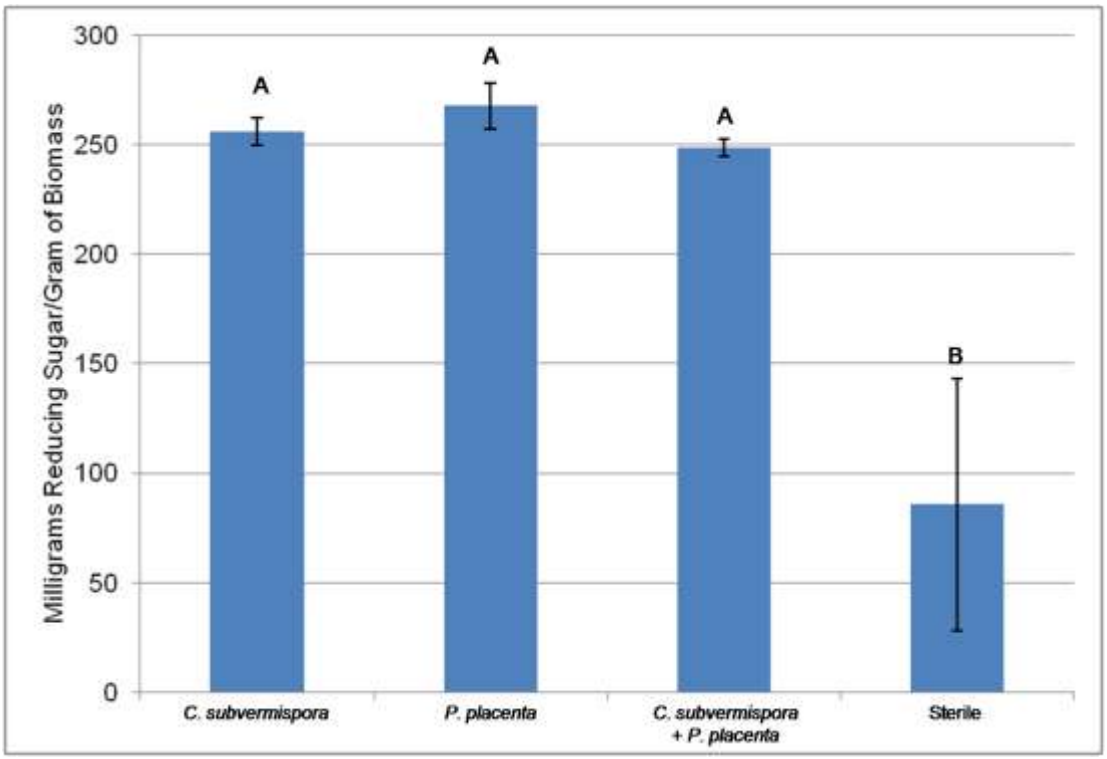

Figure 3.3: Percent reducing sugars solubilized after enzymatic hydrolysis of *Liriodendron tulipifera* . Means with the same letter were not significantly different. Error bars=1SE.

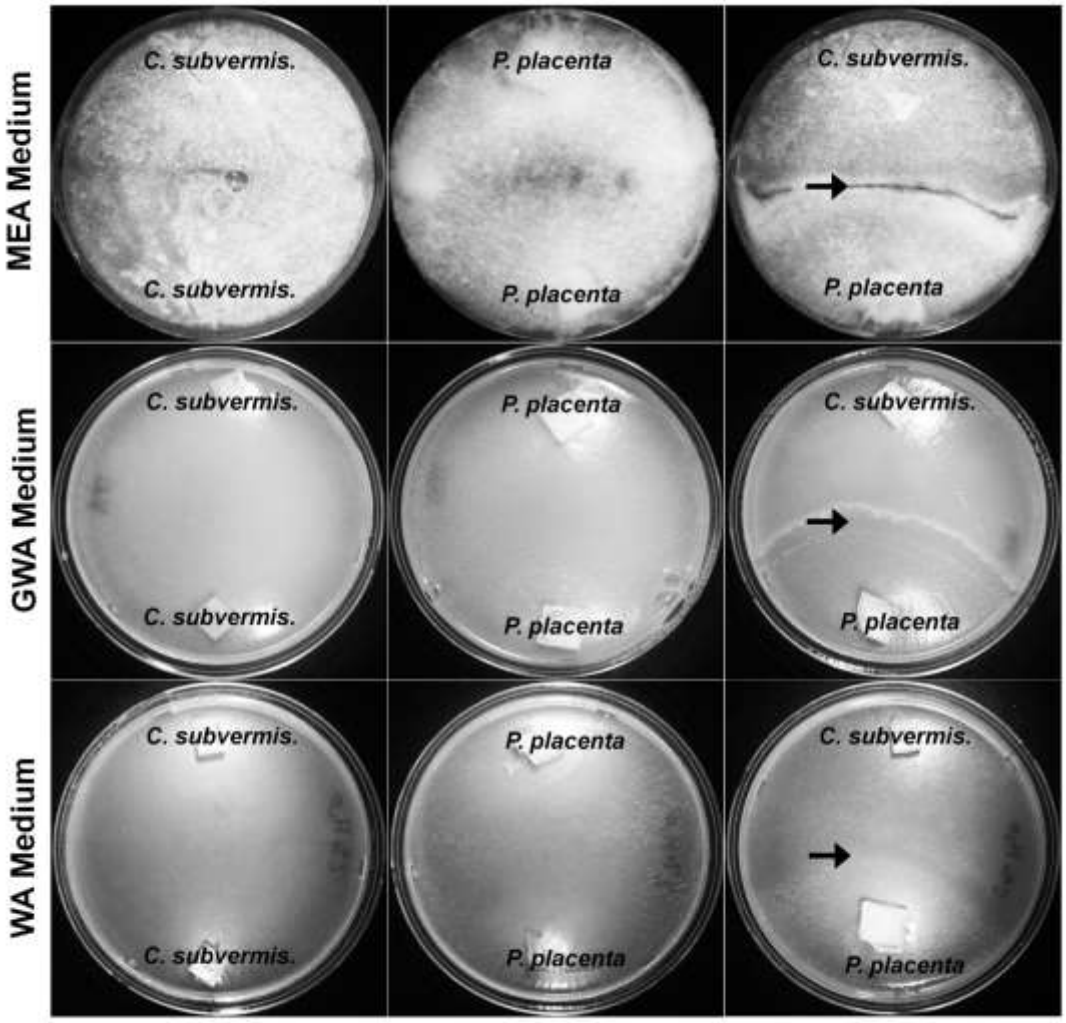

Figure 3.4: Intraspecific and interspecific interaction in cultures of *Ceriporiopsis subvermispora* and *Postia placenta* media incubated at 28° C for 30 days on malt extract agar (MEA), ground wood (*Liriodendron tulipifera)* agar (GWA), and 2% water agar (WA). Arrows denote mycelium-free zone of inhibition between opposing species.

CHAPTER 4: TWO-STAGE FUNGAL BIOPULPING SOLUBILIZES LIGNOCELLULOSIC CARBOHYDRATES WITHOUT SUPPLEMENTARY ENZYMATIC HYDROLYSIS

The following chapter will be submitted to the scientific journal *Bioresource Technology* in March 2013 and is currently being reviewed by coauthors. Giles, R.L., Galloway, E.R., Zackeru, J.C., Naithani, V., Jameel, H., and Parrow, M.W., 2013. Two Stage Fungal Biopulping Solubilizes Lignocellulosic Carbohydrates Without Supplementary Enzymatic Hydrolysis. Bioresource Technology. (In preparation).

Abstract

Novel applications of wood decay fungi were examined for effects on carbohydrate solubility before and after enzymatic hydrolysis. *Pinus taeda* wood chips were selectively decayed in solid-phase treatments of white rot (*Ceriporiopsis subvermispora* and *Pleurotus ostreatus*) and brown rot (*Postia placenta* and *Fomitopsis cajanderi*) fungi in co-culture and succession. Novel two stage fungal colonization treatments converted ca. 6.4-7.4% of the bulk biomass to reducing sugars without downstream enzymatic hydrolysis. Two stage fungal treatments utilizing novel fungal species significantly increased the yield of enzymatic hydrolysis (ca.19% increase over controls) utilizing industrial standard enzyme and solids loading. This study is the first to report significant depolymerized reducing sugars liberated from biopulped wood chips without an enzymatic hydrolysis treatment.

Introduction

Ethanol production from lignocellulosic biomass requires a chemical or enzymatic hydrolysis step to depolymerize and solubilize biomass carbohydrates (Hahn-Hägerdal et al. 2006; Froese et al. 2008). These energy intensive, low yield hydrolysis methods significantly increase the cost of lignocellulosic biomass processing, thus making woody feedstock processing more expensive than using corn or sugarcane (Hahn-Hägerdal et al. 2006). An ideal feedstock processing method would eliminate this chemical or enzymatic hydrolysis step. However due to the highly polymerized nature of cellulose imbedded within recalcitrant lignin a suitable method has not yet been developed (Hahn-Hägerdal et al. 2006; Froese et al. 2008). The overall goal of this research was to develop a fungal treatment method that would eliminate or supplement downstream enzymatic hydrolysis. In this study, we comparatively examined single and two stage applications of both laboratory and wild isolates of wood decay fungi and the effects on carbohydrate solubility before and after enzymatic hydrolysis.

Naturally occurring forest wood decay fungi that degrade lignocellulosic biomass have been used as alternative "biopulping" pretreatments for paper production, chemical remediation, and biofuels production (Blanchette 1984; Aktar et al. 1992; Scott et al. 1998; Tortella et al. 2008; Bak et al. 2009; Couto 2009; Vimala and Das 2009; Fissore et al. 2010; Rasmussen et al. 2010; Giles et al. 2011). We have previously described a two-stage biopulping method that significantly increases the glucose yield of enzymatic hydrolysis (Giles et al. 2011, 2012b). This two stage process utilizes the well characterized decay mechanisms of *Ceriporiopsis subvermispora* and *Postia placenta* to

effectively increase lignocellulosic ethanol yield (Akhtar et al. 1994; Choi et al. 2006; Wymelenberg et al. 2009; Giles et al. 2011).

Based on observations with well characterized fungal species, we propose there are fungi capable of depolymerizing and solubilizing carbohydrates within woody feedstocks without further downstream processing. Newly discovered species and strains of wood decay fungi with better select degradation capability could significantly improve current fungal biotechnologies by eliminating the need for downstream hydrolysis, however species effects have not been fully explored (Giles et al. 2012b). A hypothetical fungal pretreatment method that requires no downstream enzymatic hydrolysis would significantly improve the commercial viability of lignocellulosic ethanol production. However, very few of the abundant wild wood decay fungal species have been isolated for use in this emerging field of biotechnology and even fewer have been evaluated. In this study we utilized wild isolates of *Pleurotus ostreatus* (Order Agaricales) and *Fomitopsis cajanderi* (order Polyporales) and the well characterized wood decay fungal species *C. subvermispora* and *P. placenta* (Wymelenberg et al. 2009; Giles et al. 2011).

Two novel degradation effects that have been investigated (but not fully explored for biopulping) include applications of novel wild fungal isolates and co-culture innoculations. Co-culture applications of the lignin selective white rot fungus *C. subvermispora* and simultaneous white rot fungus *P. ostreatus* stimulate lignolytic activity increasing the decay of acid-soluble lignin (Chi et al. 2007). Cellulose degree of depolymerization, colonization growth rate, and substrate decay capability varies among brown rot fungi (Filley et al. 2002; Howell et al. 2009, Fissore et al. 2010; Rasmussen et al. 2010). Based on this previous research and preliminary testing we developed two

hypothesis. First, we hypothesized that co-inoculating *C. subvermispora* with *P. ostreatus* in a single application would increase the lignin degrading activity and increase reducing sugar solubility over previously described biopulping methods and controls. Second, we hypothesized that the wild isolate brown rot *F. cajanderi* would significantly alter the downstream reducing sugar solubility when compared to *P. placenta* treatments and controls.

Materials and Methods

Laboratory Fungal Strains

Previously described biopulping species isolates *C. subvermispora* FP-90031-sp (a lignin-selective white rot fungus), and *P. placenta* Mad-698-R (a brown rot fungus) was obtained from USDA Forest Products Laboratory, Madison, Wisconsin, USA (Giles et al. 2011). Isolates were maintained in the laboratory at 28°C in darkness on modified malt extract agar (20 g malt extract, 1.0 g yeast extract, and 20 g agar L^{-1} in deionized water) until use.

Wild Strain Collection, Isolation, and Molecular Authentication

Basidiocarps of *P. ostreatus* and *F. cajanderi* were collected in North Carolina, USA. Macroscopic and microscopic characteristics were used to identify the collected reproductive sporocarps to species using existing taxonomic monographs and treatments (Overholts 1953; Lowe 1966; Breitenbach and Kraenzlin 1986; Gilbertson and Ryvarden 1986; Gilbertson and Ryvarden 1987; Jung 1987; Vilgalys et al. 1993). Macroscopic characters such as basidiocarp type (e.g. resupinate, applanate), presence/absence of stipe, and margin growth were used for identification. Micromorphology such as context tissue hyphal types (e.g. skeletal, generative) were

identified using standard light microscopy and staining reaction techniques (e.g. Melzner's reagent) (Gilbertson and Ryvarden 1986; Gilbertson and Ryvarden 1987; Vilgalys et al. 1993; Giles et al. 2008). Tissue samples from context tissue (the sporocarp was split and sterile tissue removed) was placed on non-selective fungal media (20 g malt extract, 1.0 g yeast extract, and 20 g agar L^{-1} in deionized water and amended with 250 µg streptomycin sulphate/mL to prevent bacterial growth) and incubated at 25°C for 7-10 days (Clausen and Kartal 2003; Ferraz et al. 2003; Hundorf et al. 2004; Yang 2005; Giles 2008). The colonies were then subcultured onto new agar plates to grow as pure cultures. All wild isolates were maintained in the laboratory (conditions were identical to those used for laboratory isolates) at 28°C in darkness on modified malt extract agar (20 g malt extract, 1.0 g yeast extract, and 20 g agar L^{-1} in deionized water).

Fungal DNA was extracted from each isolate using modified previously described techniques (Lõoke et al. 2011; Fawcett and Parrow 2012). Approximately 100 mg of fungal mycelium was transferred to a 2mL microcentrifuge tube. 200µL of 200 mM lithium acetate (LiOAc) with 1% sodium dodecyl sulfate (SDS) was added and incubated at 70°C for 15 minutes. The fungal mycelia was mechanically ground using a glass rod until the sample was homogenized. After incubation, 300µL of 95% ethanol was added to the reaction mixture, vortexed for approximately 15 seconds, and centrifuged for 5 minutes at 15,000 relative centrifugal force (rcf). The supernatant was removed and 500 µL of 70% ethanol was added. The sample was then mixed by vortexing for approximately 30 seconds and centrifuged for 5 minutes at 15,000 relative centrifugal force (rcf). The supernatant was removed and the sample was resuspended in 100 µL of H_2O. Nanodrop spectroscopy was used to verify DNA concentrations before conducting

PCR. Amplification of the ITS region of the rDNA was performed using modified previously described techniques and primers (White et al. 1990; Zervakis et al. 2004; Priyadarsini et al. 2011). The rDNA ITS regions 1 and 2 were amplified using ITS1-F (sequence: CTTGGTCATTTAGAGGAAGTAA) and ITS4-B (sequence: CAGGAGACTTGTACACGGTCCAG) primers. These primers have been previously observed to amplify the entire ITS region and show specificity towards Basidiomycetes (Gardes and Bruns 1993). The PCR products were observed using gel electrophoresis on a 1% agarose gel prepared in 1x Tris-acetate-EDTA (TAE) buffer. The agarose gels were stained with 0.5µg/mL ethidium bromide solution for 30 minutes before being washed with deionized water. DNA bands were visualized under an ultra-violet transilluminator and then photographed. Sequencing was performed by Applied Biosystems BigDye version 3.1 (GENEWIZ, Inc., South Plainfield, NJ, USA) (Fawcett and Parrow 2012). Sequences were queried using an advanced, nongapped NCBI BLAST search with expectation frequency set to 0.0001 and no filtering for low complexity as previously described (Henry et al. 2000). The highest bit score and maximum identity similarity percentage listed in the BLAST results search were used to verify morphological identification (Altschul et al. 1997).

Wood, Treatments, and Culture Conditions

Fresh cut Loblolly Pine *(Pinus taeda)* wood chips were collected from a local hardwood lumber mill and stored at 4°C until use. The wood was a mixture of earlywood and latewood chipped from duramen without orientation. The chips were visually inspected to remove bark pieces or defect wood and to verify the homogeneity of all

samples before treatment. Chips were flat in shape and approximately 2-3 cm in diameter.

Single and two stage biopulping was performed using modification of previously described techniques (Giles et al. 2011, 2012a, 2012b). Prior to inoculation onto wood, fungal isolates were subcultured in low nitrogen malt extract agar (20 g malt extract, 20 g agar L^{-1} in deionized water) at 28°C for 7-10 d. A fungal plug from the malt extract agar plate of each species was then placed in 50 mL of low nitrogen malt extract liquid medium (20 g malt extract L^{-1} in deionized water) and incubated at 28°C for 20 d. For each treatment, 34 g (oven dry weight) of *P. taeda* wood chips was placed into 100 ml glass culture bottles and distilled water was added to increase moisture content (water volume adjustments were made for each treatment to reach 70% moisture content after inoculation). The loosely capped vials were then steam sterilized for 30 min. The uninoculated controls were also sterilized.

The liquid fungal cultures were vigorously shaken for one min before aseptically vacuum filtering (0. 2µm filter) to separate mycelia from media and rinsed with deionized water. The mycelia was then transferred to 100 mL deionized water and shaken one minute to homogenize. The nutrient free mycelial slurry was used to aseptically inoculate each wood chip treatment vial. Single treatments were inoculated with 8 mL of the mycelia slurry (either *C. subvermispora*, *F. cajanderi*, *P. ostreatus* or *P. placenta)*. Co-culture treatments were inoculated with 8 mL of both *C. subvermispora* and *P. ostreatus.* All treatments and controls were conducted in triplicate. All vials were incubated for 30 d at 28°C for optimal delignification and depolymerization (Clausen and Kartal 2003; Ferraz et al. 2003; Giles et al. 2011). After the 30 d treatment period, all

culture bottles (including uninoculated controls) were steam sterilized for 30 min to halt biological activity. The bottles designated for two stage biopulping were then inoculated with the second fungal species (either *F. cajanderi* or *P. placenta*), using the same inoculation volume and sterile controls as described above. The two stage treatment culture bottles were then incubated for an additional 30 d at 28°C followed by steam sterilization for 30 min.

Chemical Analyses of Wood

Gravimetric determination of wood holocellulose, α-cellulose, and Klason lignin content of the sterile controls and biopulped samples was performed using previously described microanalytical techniques (Yokoyama et al. 2002; Yeh et al. 2004; Sluiter et al. 2008; Giles et al. 2011). Wood was ground into meal (40 mesh particle size), oven dried at 104°C, weighed, and then extracted using a Sohxlet apparatus to wash 95% EtOH over the samples for 24 h. The extracted material was then oven dried at 104°C.

For each treatment, holocellulose was quantified from 100 mg of oven dry weight extractive free wood meal. Each sample was placed into a 20 ml round bottom flask and 4 mL of deionized water, 200 mg of 80% sodium chlorite, and 0.8 mL of glacial acetic acid was added. The flasks were then capped and submerged in a 90°C water bath for 1 h. The flasks were then cooled and their contents filtered using a sintered glass filter (medium pore size). The holocellulose filtrate was washed with deionized water and dried at 104°C before weighing. Holocellulose contents were recorded as a percentage of original sample mass.

α-cellulose was isolated from 50 mg of isolated holocellulose from each treatment. The holocellulose was treated with 4 mL of 17.5% sodium hydroxide for 30

min, and the reaction was then diluted with 4 mL of deionized water and incubated for 30 minutes. The resultant α-cellulose was collected by filtration using a sintered glass filter (medium pore size), washed with deionized water, and dried at 104°C before weighing. α-cellulose contents were recorded as a percentage of original sample mass.

Klason lignin was prepared from extractive free wood meal. For each sample, 300 mg of wood (oven dry weight) and 3 mL of 72% sulfuric acid was placed in a 90 mL pressure tube. The sample was stirred with a glass rod every 15 min for 60 min, and then diluted to 4% concentration by addition of 84 mL of deionized water. The pressure tubes were then capped and autoclaved for one hour at 121°C, stored overnight at 4°C, and filtered using a sintered glass filter (medium pore size). The acid insoluble lignin filtrate was then washed with deionized water and dried at 104°C before weighing. Klason lignin contents were recorded as a percentage of original sample mass.

Enzymatic Hydrolysis and Determination of Sugar Yield

Enzymatic hydrolysis of treatment and control samples was conducted following the methods of Shi et al. (2009) and Giles et al. (2011, 2012b) on ground material (40 mesh particle size) from triplicate treatments and controls. Cellulase (40 FPU/g of substrate), β-glucosidase, and hemicellulase (Cellic CTec2, Novozyme Co.) with a 5% biomass loading volume was used (3 replicates with enzyme hereto referred to as "hydrolysis liquor" and 3 replicate controls without enzyme per biopulping treatment hereto referred to as "buffer solution"). Samples were shaken at 50°C for 72 h and then placed in a boiling water bath for 10 minutes to halt saccharification. Samples were then centrifuged for 10 min at 1200 g. Supernatants were stored at 4°C until use. Samples were analyzed using the Somogyi-Nelson method for colorimetric determination of reducing sugars

(Fournier 2001). Sugar quantification was performed using dilutions of a glucose stock standard.

Agar Plate Assays for Interspecific Growth Interactions

Fungal growth interactions were examined by spot-inoculating agar Petri plates on opposite sides (approximately 40mm apart) of the assay plate in a pairwise matrix fashion (e.g. *C. subvermispora* versus *C. subvermispora*, *P. ostreatus* versus *P. ostreatus*, or *C. subvermispora* versus *P. ostreatus*). Square agar plugs ($1cm^2$) cut from stock malt extract agar plate cultures were used to aseptically inoculate each assay plate at opposing sides. To examine the potential effects of substrate type or availability on growth interactions, two different assay media types were used: 1) ground wood agar (GWA) (20 g of 40 mesh ground *P. taeda* wood, 20 g agar L^{-1} in deionized water; and 2) 2% water agar (WA) (20 g agar L^{-1} in deionized water). All treatment combinations and sterile controls were conducted in triplicate. The assay plates were incubated at 28°C in darkness for 30 d to allow adequate time for the fungal mycelia to spread across the surface and meet in the middle of each plate, and observed for gross interactions at the central convergence zone between the two opposing mycelial growth fronts. Interactions between opposing mycelia were assessed visually using the classification methods previously described (Rayner and Boddy 1988). Plates were observed again after 60 d to assess longer-term interactions.

Statistical Analysis

To test the effect of fungal treatments on wood chemical properties and soluble reducing sugars, all measured values were analyzed using One-way ANOVA (α=0.05) in JMP® 9.0.2 (SAS Institute, Cary, North Carolina, USA). Tukey-Kramer tests were used

to determine significant differences between controls and treatments, and among different treatments ($\alpha = 0.05$).

Results and Discussion

Identification of Novel Fungi

Molecular identification results authenticated morphological classification of the wild isolates (Altschul et al. 1997). A maximum identity similarity of 100% between the wild isolate of *P.ostreatus* and the American Type Culture Collection (Virginia, USA) isolate *P. ostreatus* ATCC 38539 was observed. A maximum identity similarity of 99% between the wild isolate of *F. cajanderi* and a referenced *F. cajanderi* isolate was observed (Kim et al. 2007).

Biopulping Effects on Chemical Composition

In all fungal treatments, mycelial mats were observed on the wood chips within 15 days of inoculation, suggesting rapid and complete colonization of the wood chips. Post-treatment wood chemical compositions were reported as percentages of final mass chemical composition, not percent component degradation. Comparisons between single and two stage sterile controls suggest biomass sterilization decreases the final holocellulose composition percentage in *P. taeda* (Table 4.1). This decrease was not previously observed in autoclaved *Liriodendron tulipifera* wood chips suggesting that polysaccharides in *P. taeda* wood chips are more easily solubilized by pressure and heat (Giles et al. 2011). In all single and two stage treatments and controls, Klason lignin content was not significantly reduced (Table 4.1). This was expected; previous observations suggest total bulk acid insoluble lignin percentage is not reduced by single stage *C. subvermispora*, *P. placenta*, co-culture treatments of *C. subvermispora* and *P.*

ostreatus, or two stage treatments in succession (Chi et al. 2007; Giles et al. 2011; Giles et al. 2012a, 2012b).

The holocellulose contents exhibited significant differences between the treatments and controls suggesting fungal modification of biomass composition percentages (Table 4.1). Within single stage treatments, the holocellulose content in the *F. cajanderi* treated wood was significantly reduced when compared to the single stage control and the *C. subvermispora* treatment (Table 4.1). The *F. cajanderi* fungus degraded approximately 15% of the total *P. taeda* polysaccharides within the short 30-day colonization period. *P. placenta* treated wood did not exhibit significant holocellulose loss when compared to controls (Table 4.1). This supports previous observations of species specific degradation characteristics and may also be mediated by substrate (Gilbertson and Ryvarden 1986; Gibertson and Ryvarden 1987; Howell et al. 2009). Brown rot fungi rapidly depolymerize anamorphous and semi-crystalline cellulose by utilizing peroxides (and a diminutive quantity of cellulases), cleaving the long carbohydrates into shorter chains and increasing the microfibril permeability, while leaving a modified demethylated lignin behind (Eriksson et al. 1990; Filley et al. 2002; Kleman-Leyer et al. 1992; Irbe et al. 2006). Dissimilar holocellulose degradation was not expected since both *F. cajanderi* and *P. placenta* are brown rot fungi typically found on fallen Gymnosperm wood in the forest however, the differences in holocellulose degradation may also be due to intraspecies strain variability not examined in this study (Gilbertson and Ryvarden 1986; Gibertson and Ryvarden 1987; Grand and Vernia 2006; Kang et al. 2009). *F. cajanderi* biomass degradation has not been fully characterized

therefore further study of this species will elucidate the mechanisms of holocellulose decay and the capability for biotechnological applications.

Holocellulose and lignin contents did not exhibit significant differences between the two stage treatments or controls (Table 4.1). Unexpectedly, the holocellulose content did not decrease among the two stage fungal treatments. The novel two stage *C. subvermispora* + *P. ostreatus*/*P. placenta* and *C. subvermispora*/*F. cajanderi* treatments were similar to sterile wood suggesting no increase in carbohydrate loss when compared to controls or single stage applications (Table 4.1). It is important to note that a single stage application of *F. cajanderi* induced holocellulose loss, but a successional two stage application of *C. subvermispora* followed by *F. cajanderi* did not exhibit polysaccharide loss (Table 1). This unexpected result suggests potential interaction between the previously *C. subvermispora* colonized material and *F. cajanderi* not previously observed with *P. placenta* (Giles et al. 2011). The biomass was steam sterilized after the initial *C. subvermispora* colonization stage; therefore the interaction is either substrate or small molecule mediated. It is possible that *C. subvermispora* alters the substrate thus causing *F. cajanderi* to reduce growth or degradative capability though an unknown substrate interaction mechanism (Rayner and Boddy 1988). Alternatively, *C. subvermispora* may leave heat resistant small signal molecules which alter the growth patterns of *F. cajanderi* (Palkova et al. 1997; Nigram et al. 2011; Cotier and Muḧlschlegel 2012).

The holocellulose and lignin contents of the *C. subvermispora* + *P. ostreatus* co-culture treatments were similar to sterile wood suggesting no increase in degradation capability of the co-culture treatment when compared among examined fungal treatments (Table 1). Co-culture applications of *C. subvermispora* + *P. ostreatus* have been

previously shown to stimulate degradation of acid soluble lignin however, no decrease in acid insoluble Klason lignin percentage was observed in this study (Chi et al. 2007) (Table 4.1). No significant effects observed in the co-culture treatments may have been caused by unknown mechanisms of interspecific fungal competition (Rayner and Boddy 1988; Chi et al. 2007; Cotier and Muḧlschlegel 2012).

The plate assay observations also suggest that these fungal interactions are substrate specific (Figure 4.1). *C. subvermispora* and *P. ostreatus* quickly colonized ground wood agar (GWA) and water agar (WA) within 15 days (with similar colonization rates). Intraspecific competiton was not observed in the same-species assays (i.e. *C. subvermispora* versus *C. subvermispora*; *P. ostreatus* versus *P. ostreatus*) (Figure 1). A substrate-specific interaction was observed amongst the dual-species assays ((i.e. *C. subvermispora* versus *P. ostreatus)* (Figure 4.1). Within the duel-species assays, *C. subvermispora* and *P. ostreatus* mycelia merged at the center of the agar and no growth inhibition was observed (Figure 4.1). Upon stereoscopic observations, neither fungus exhibited dominance or replacement growth patterns, suggesting both can grow within close proximity. Substrate specific characterization must be performed to determine if simultaneous co-culture applications of white rot fungi are beneficial for lignocellulosic ethanol biomass pretreatment.

Evaluation of Novel Biopulping Species for Lignocellulosic Ethanol Production

An approximate 37-41% conversion of biomass to reducing sugars after enzymatic hydrolysis was observed in both single stage controls and fungal treatments (Table 4.2). Single staged, single-species fungal treatments did not significantly increase the yield of reducing sugars produced in enzymatic hydrolysis when compared to the

control untreated wood (Table 4.2). This was expected as recent research has suggested single applications of biopulping fungi show little improvement of wood enzymatic hydrolysis (Rasmussen et al. 2010; Giles et al. 2012b).

A significant increase in hydrolysis yield (ca. 19% increase over controls) was observed in the two stage fungal treatments utilizing novel fungi *P. ostreatus* and *F. cajanderi* (Table 4.2). The observed carbohydrate increase suggests novel fungi can be utilized to advance previously described biopulping methods, therefore further characterization of wild isolates should be conducted (Giles et al. 2011). It is important to note that none of the fungal treatments exhibited an inhibitory effect on enzymatic hydrolysis efficiency (Table 4.2). The *F. cajanderi* treatments reduced overall holocellulose content but increased carbohydrate solubilization (Tables 4.1 and 4.2). This suggests the fungus grew primarily on the non-fermentable hemicelluloses within the wood.

Unexpectedly in two stage fungal treatments, we observed significant increase in reducing sugars within buffered solutions without cellulase which were not observed in controls or single stage fungal applications (Table 4.2). This result supports a previously described additive effect of two staged white rot lignin removal and brown rot cellulose depolymerization on hydrolyzed wood (Giles et al. 2011). However, this study is the first to report significantly increased reducing sugars within a buffer solution without supplemental depolymerization using enzymatic hydrolysis. The two stage *C. subvermispora/P. placenta*, *C. subvermispora* + *P. ostreatus/P. placenta,* and *C. subvermispora*/*F. cajanderi*, treatments exhibited an approximate 6.5-7.5% conversion of total bulk biomass to reducing sugars without enzymatic hydrolysis (Table 4.2). These

observed soluble reducing sugars have the potential to supplement commercial lignocellulosic ethanol production by providing a relatively simple method of producing fermentable carbohydrates. This novel method of utilizing both white and brown rot fungi degradation capability without enzymatic hydrolysis could significantly improve current lignocellulosic biotechnologies. Soluble fermentable reducing sugars removed during low cost/energy rinsing could reduce the cost of downstream enzymatic hydrolysis by reducing or eliminating the need for enzyme loading (Figure 4.2).

The effect of biomass type, cellulase loading, biomass drying, and fungal species on biopulping needs exploration in order to increase soluble monomeric carbohydrate yield. Prior two stage biopulping studies have focused on hardwood *L. tulipifera* wood chips which significantly differ in anatomy and cell wall chemical composition from *P. taeda* (Giles et al 2011, 2012b). In addition, the unknown effects of cellulase loading volume on carbohydrate solubilization must be further characterized in order to further optimize the cost and speed of a biopulping process for commercial applications. Previous research also suggests the pores developed during fungal decay may collapse during oven drying, therefore reducing the effects of mechanical degradation (Tanaka et al. 1988; Wong et al. 1988; Mansfield et al. 1999). Due to the abundant literature characterizing *C. subvermispora* and *P. placenta*, most research utilizing white and brown rot fungi for biotechnological applications has focused on these two species. However, these two species are only a small representation of the biological diversity within the decay fungi.

Select fungal strains exhibiting rapid growth and secretomes (such as those used in previous biopulping studies) have been widely used in studies of wood decay and

fungal degradation biotechnologies (Aktar et al. 1992; Scott et al. 1998; Choi et al. 2006; Lee et al. 2008; Mendonça et al. 2008; Giles et al. 2011, 2012a, 2012b). These particular strains have been sub-cultured extensively and in some cases, the original dried specimen no longer exists for study (as in the case of *C. subvermispora* FP-90031-sp and *P. placenta* Mad-698-R). These two fungal strains were originally collected in 1960 and 1941 respectively (USDA FPL 2010). Although storage in liquid nitrogen has been shown to reduce genetic instability, this method of preservation is not optimal for all fungal species (Arora et al. 2004; Ryan and Smith 2004). It has also been observed that the continuous sub-culturing of select isolates from culture collections can result in less than optimal growth patterns (Arora et al. 2004, Ryan and Smith 2004). Notably, repeated sub-culturing of fungal strains also leads to alteration of fungal morphology, reproductive cycles, and virulence however the mechanisms are unclear (Kistler and Miao 1992, Kamp and Bidochka 2002, Ryan and Smith 2004).

Possibly more significant than culture collection conditions, geographical region and host substrate has been shown to generate fungal strains within species that exhibit different decay characteristics (Hakala et al. 2004; Wolfaardt et al. 2004; Howell et al. 2009). Strain variation within species has been observed in applied biopulping and wood decay resistance studies using geographically separated isolates of Polyporales species *Ceriporiopsis* and *Ganoderma* (Aktar et al. 1992; Scott et al. 1998; Hakala et al. 2004; Mendonça et al. 2008). Unfortunately, very few total fungal species (even fewer isolated strains) have been described globally. Only 100,000 species of fungi have been classified to date; leaving 95% of the total global estimated 1.5 million species undiscovered (Hawksworth 1991, Heywood 1995, Rossman 1995, Hawksworth 2001). Of described

species, only approximately 2,000 of described species are North American wood decay fungi, while even fewer have been studied for decay capability (the Order Polyporales contains *C. subvermispora*, *F cajanderi*, and *P. placenta* used in this study) (Gilbertson 1984, Hawksworth 2001, Kirk et al. 2001). In our study, we examined only two isolates collected from forays in North Carolina. Further exploration of understudied wood decay fungi capability is required to develop a low cost efficient method of biopulping for ethanol applications.

Conclusions

We have demonstrated that wild isolated species of wood decay fungi can significantly improve current lignocellulosic ethanol technologies. These novel fungal species isolates were specifically examined to supplement or replace standard species used in fungal biopulping for lignocellulosic ethanol applications. The examined species were capable of liberating significant reducing sugars both with and without downstream enzymatic hydrolysis. Multi-staged, whole-organism wood decay fungal treatments for lignocellulosic ethanol production could improve cost-efficiency of current commercial purified enzymatic treatments thus addressing the cost-prohibitive nature of lignocellulosic ethanol.

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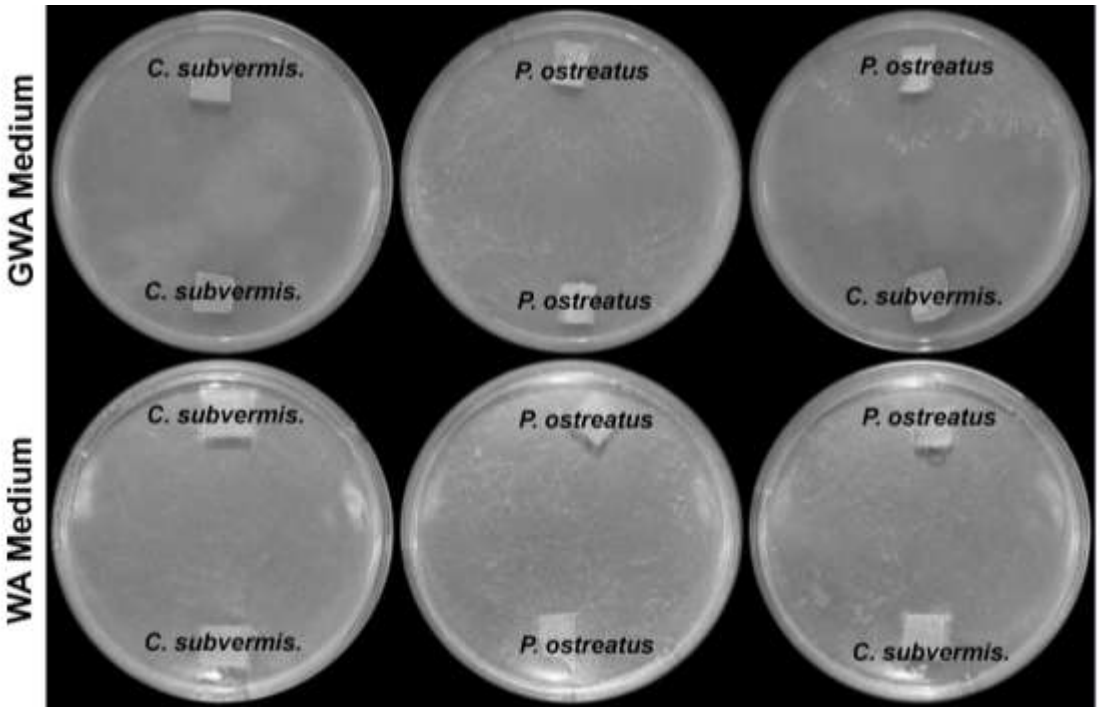

Figure 4.1: Intraspecific and interspecific interaction in cultures of *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* media incubated at 28° C for 30 days on ground wood (*Pinus taeda)* agar (GWA) and 2% water agar (WA).

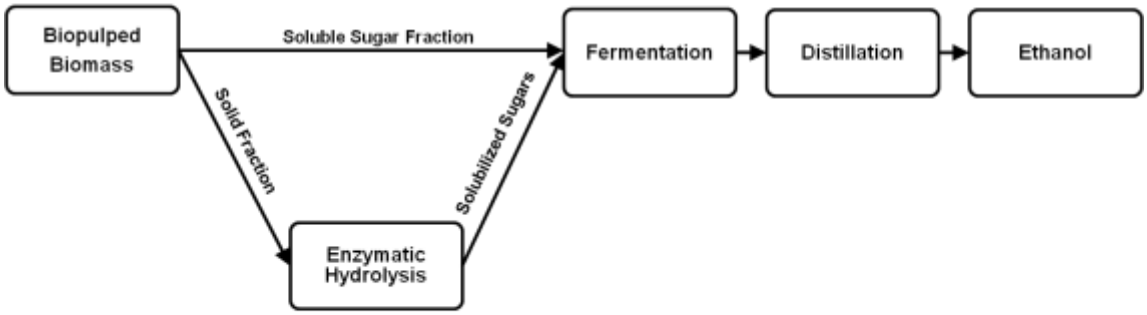

Figure 4.2: Bioethanol production process diagram. Solubilized sugars liberated during a biopulping pretreatment process could be utilized in downstream fermentation.

Table 4.1: Chemical composition of *Pinus taeda* wood chips after degradation, means ± 1SE, n=3. Means with the same superscript letter were not significantly different. The number of sterilizations is denoted by the treatment superscript.

Treatment	N	Hollocellulose % ±SE	Klason Lignin % ±SE
Sterile[1]	3	79.14 ±1.85^{A}	28.56 ±0.28^{A}
C. subvermispora[1]	3	66.33 ±2.94^{A}	31.29 ±5.44^{A}
P. ostreatus[1]	3	78.00 ±3.93^{AB}	26.68 ±1.13^{A}
P. placenta[1]	3	73.78 ±.77^{ABC}	28.02 ±1.28^{A}
C.subvermispora + *P. ostreatus*[1]	3	72.08 ±3.13^{ABCD}	26.93 ±1.89^{A}
F. cajanderi[1]	3	67.21 ±2.58^{BCD}	28.79 ±0.55^{A}
Sterile[2]	3	65.38 ±2.69^{CD}	26.27 ±1.02^{A}
C. subvermispora followed by *P. placenta*[2]	3	61.83 ±0.92^{D}	25.95 ±1.42^{A}
C.subvermispora + *P. ostreatus* followed by *P. placenta*[2]	3	72.80 ±0.14^{ABCD}	27.83 ±2.84^{A}
C. subvermispora followed by *F. cajanderi*[2]	3	69.81 ±1.47^{ABCD}	27.83 ±1.80^{A}

Table 4.2: *Pinus taeda* wood chips converted to reducing sugars, means ± 1SE, n=3. Means with the same superscript letter were not significantly different. The number of sterilizations is denoted by the treatment superscript.

Treatment	N	Buffer Solution	Hydrolysis Liquor
		Biomass% Converted to Reducing Sugars ±SE	Biomass% Converted to Reducing Sugars ±SE
Sterile[1]	3	0.66 ±0.35^{C}	37.25 ±0.68^{AB}
C. subvermispora[1]	3	2.65 ±0.23^{C}	37.93 ±0.30^{AB}
P. ostreatus[1]	3	0.95 ±0.22^{C}	40.62 ±0.50^{A}
P. placenta[1]	3	1.88 ±0.66^{C}	39.02 ±0.79^{AB}
C.subvermispora + *P. ostreatus*[1]	3	0.80 ±0.01^{C}	41.12 ±1.91^{A}
F. cajanderi[1]	3	3.19 ±0.82^{BC}	39.29 ±1.06^{AB}
Sterile[2]	3	0.67 ±0.07^{C}	34.37 ±2.11^{B}
C. subvermispora followed by *P. placenta*[2]	3	6.42 ±1.79^{AB}	39.11 ±0.26^{AB}
C.subvermispora + *P. ostreatus* followed by *P. placenta*[2]	3	6.60 ±0.76^{AB}	41.65 ±0.78^{A}
C. subvermispora followed by *F. cajanderi*[2]	3	7.44 ±0.58^{A}	41.21 ±0.75^{A}

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APPENDIX A: LIGNOCELLULOSIC TREATMENTS AND APPLICATIONS THEREOF

The following was submitted to United States Office of Patents in 2011 and is currently pending in review. Giles, R., Parrow, M., 2011. Lignocellulosic Treatments And Applications Thereof. U.S. Patent. 20110294169. Filed May 2011. Patent Pending.

RELATED APPLICATION DATA

The present application hereby claims priority pursuant to 35 U.S.C. § 119(e) to United States Provisional Patent Application Serial Number 61/348,278, filed May 26, 2010, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to treatments for decomposing lignocellulosic materials and, in particular, to the use of whole microorganisms in the decomposition process.

BACKGROUND

Lignocellulosic materials are widely abundant sparking considerable interest in these materials for various biofuel applications. Lignocellulosic biomass such as wood waste, crop stalks and grasses are potentially sustainable sources of biomass for ethanol production. With most of the terrestrial biomass on earth being lignocellulosic, producing ethanol from lignocellulosic material has the potential to replace up to 30% of annual petroleum consumption in the United States while significantly reducing greenhouse gas emissions. Moreover, the use of lignocellulosic material in ethanol

production does not encounter food production pressures as with other crop sources for ethanol such as corn.

Nevertheless, the abundant sugars contained in lignocellulosic materials are blocked from traditional ethanol-producing fermentation reactions because they typically occur in a complex polymerization of lignin and celluloses that is difficult to hydrolyze into soluble sugars for fermentation. Since lignin is highly resistant to water penetration and enzymatic breakdown, it represents a significant barrier to isolating cellulose and other sugars for use in production of both paper and ethanol biofuel. Similarly, the high degree of polymerization of cellulose in woody biomass is also a significant barrier to efficient biofuel conversion and requires chemical and/or enzymatic hydrolysis to produce soluble sugars for fermentation.

Current methods using chemical and enzymatic processes for lignin removal and cellulose hydrolysis are cost prohibitive and inefficient to support industrial-scale lignocellulosic ethanol production. Such chemical and enzymatic processes include harsh liquid-phase acid or base-catalyzed pretreatments aimed at making the cellulose more accessible to enzymatic hydrolysis. These chemical treatments additionally require specialized facilities for safely handling and disposing of hazardous chemicals, resulting in increased costs and environmental concerns. Furthermore, current enzymatic cellulose hydrolysis processes use expensive extracted and purified cellulase enzymes applied to the lignocellulosic biomass in liquid-phase batches. As a result of these significant processing drawbacks, commercially viable ethanol production from lignocellulosic materials remains unrealized.

SUMMARY

In one aspect, methods of treating lignocellulosic materials are described herein. In some embodiments, a method of treating lignocellulosic materials described herein can overcome one or more of the foregoing disadvantages of using lignocellulosic materials in the production of ethanol.

In some embodiments, a method of treating a lignocellulosic material comprises degrading lignin of the lignocellulosic material with at least one fungus and hydrolyzing cellulose of the lignocellulosic material with at least one microorganism. In some embodiments, lignin is selectively degraded by the at least one fungus. In some embodiments, the at least one fungus comprises a white rot fungus. In some embodiments, the at least one fungus comprises a plurality of fungi. The plurality of fungi, in some embodiments, comprises white rot fungi.

In some embodiments, the at least one cellulose hydrolytic microorganism of the treatment comprises at least one fungus. In some embodiments, for example, a microorganism comprises a brown rot fungus. The at least one microorganism, in some embodiments, comprises a plurality of fungi. In some embodiments, a plurality of fungi serving as the cellulose hydrolytic microorganism comprises brown rot fungi. Alternatively, a microorganism, in some embodiments, comprises one or more cellulose-hydrolytic bacteria. In some embodiments, the at least one microorganism comprises a combination of fungus and cellulose-hydrolytic bacteria.

In some embodiments, a lignin degrading fungus is applied to the lignocellulosic material prior to application of the at least one cellulose hydrolytic microorganism. In some embodiments, the microorganism is applied to the lignocellulosic material prior to application of a lignin degrading fungus. In some embodiments, a lignin degrading

fungus and a microorganism are applied to the lignocellulosic material simultaneously or substantially simultaneously.

In some embodiments wherein a lignin degrading fungus is applied to the lignocellulosic material, the lignocellulosic material is sterilized or substantially sterilized prior to application of the at least one cellulose hydrolytic microorganism. In some embodiments, for example, a lignin selective white rot fungus is applied to the lignocellulosic material for a desired period of time. The lignocellulosic material is subsequently sterilized or substantially sterilized prior to application of a microorganism, such as a brown rot fungus, for the hydrolysis of cellulose of the lignocellulosic material.

In some embodiments, the lignocellulosic material is not sterilized prior to application of the at least one microorganism such that the lignin degrading fungus and the cellulose hydrolytic microorganism exist simultaneously on the lignocellulosic material.

In some embodiments, a lignin selective fungus or fungi for use in one or more methods described herein degrades at least a portion of lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material. In some embodiments, a lignin selective fungus degrades all or substantially all of the lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material. In some embodiments, degradation of the lignin provides increased access to the cellulose and/or hemicellulose of the lignocellulosic material for hydrolysis by the microorganism.

In another aspect, methods of producing ethanol are described herein. In some embodiments, a method of producing ethanol comprises providing a lignocellulosic

material, degrading lignin of the lignocellulosic material with at least one fungus, hydrolyzing cellulose of the lignocellulosic material with at least one microorganism, further hydrolyzing the cellulose with one or more cellulase enzymes into one or more fermentable sugars and fermenting the one or more sugars into ethanol.

In some embodiments, the lignin of the lignocellulosic material is selectively degraded by the at least one fungus. In some embodiments of methods of producing ethanol, the at least one fungus comprises a white rot fungus. In some embodiments, the at least one fungus comprises a plurality of fungi. The plurality of fungi, in some embodiments, comprises white rot fungi.

In some embodiments, the at least one cellulose hydrolytic microorganism of a method of producing ethanol comprises at least one fungus. In some embodiments, for example, a microorganism comprises a brown rot fungus. The at least one microorganism, in some embodiments, comprises a plurality of fungi. In some embodiments, a plurality of fungi serving as the cellulose hydrolytic microorganism comprises brown rot fungi. Alternatively, a microorganism, in some embodiments, comprises one or more cellulose hydrolytic bacteria. In some embodiments, the at least one microorganism comprises a combination of fungus and cellulose hydrolytic bacteria.

In some embodiments of producing ethanol, a lignin degrading fungus is applied to the lignocellulosic material prior to application of the at least one microorganism. In some embodiments, the microorganism is applied to the lignocellulosic material prior to application of a lignin degrading fungus. In some embodiments, a lignin degrading fungus and a cellulose hydrolytic microorganism are applied to the lignocellulosic material simultaneously or substantially simultaneously.

In some embodiments of producing ethanol wherein a lignin degrading fungus is applied to the lignocellulosic material prior to application of at least one microorganism, the lignocelluosic material is sterilized or substantially sterilized prior to application of the at least one microorganism. In some embodiments, for example, a lignin selective white rot fungus is applied to the lignocellulosic material for a desired period of time. The lignocellulosic material is subsequently sterilized or substantially sterilized prior to application of a microorganism, such as a brown rot fungus, for the hydrolysis of cellulose of the lignocellulose material.

In some embodiments, the lignocellulosic material is not sterilized prior to application of the at least one microorganism such that the lignin degrading fungus and the cellulose hydrolytic microorganism exist simultaneously on the lignocellulosic material.

In some embodiments of producing ethanol, a lignin selective fungus or fungi degrades at least a portion of lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material. In some embodiments, a lignin selective fungus or fungi degrades all or substantially all of the lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material.

In some embodiments, degradation of the lignin provides increased access to the cellulose and/or hemicellulose of the lignocellulosic material for hydrolysis by the microorganism and one or more cellulase enzymes. As described further herein, hydrolysis of cellulose by the microorganism and one or more cellulase enzymes can produce sugars suitable for fermentation into ethanol by techniques known to one of skill

in the art. In some embodiments of producing ethanol, sugars produced according to methods described herein comprise hexoses, pentoses and/or mixtures thereof. In some embodiments, hexoses comprise glucose, mannose or galactose or mixtures thereof. In some embodiments, pentoses comprise xylose or arabinose or mixtures thereof.

In another aspect, bioreactors for treating lignocellulosic material are described herein. In some embodiments, a bioreactor comprises a container for storing lignocellulosic material and apparatus for introducing at least one lignin degrading fungus and at least one cellulose-hydrolytic microorganism to the lignocellulosic material in the container. In some embodiments, a lignin degrading fungus and cellulose-hydrolytic microorganism introduced to the lignocellulosic material through the apparatus of the bioreactor can comprises any of the same described herein.

Moreover, in some embodiments, a bioreactor described herein further comprises one or more agitators for mixing or mechanically agitating the lignocellulosic material in the container. Additionally, in some embodiments, a bioreactor described herein comprises apparatus suitable for autoclaving or otherwise sterilizing the lignocellulosic material in the container.

These and other embodiments are described in further detail in the detailed description which follows.

BRIEF DESCIRPTION OF THE DRAWINGS

Figure 1 illustrates the lignin content of untreated wood and wood treated with a lignin selective fungus according to some embodiments of methods described herein.

Figure 2 illustrates percent lignocellulosic biomass converted to glucose according to methods described herein in comparison with other treatment methods.

DETAILED DESCRIPTION

Embodiments described herein can be understood more readily by reference to the following detailed description and examples and their previous and following descriptions. Elements, apparatus and methods described herein, however, are not limited to the specific embodiments presented in the detailed description and examples. It should be recognized that these embodiments are merely illustrative of the principles of the present invention. Numerous modifications and adaptations will be readily apparent to those of skill in the art without departing from the spirit and scope of the invention.

In one aspect, methods of treating lignocellulosic materials are described herein. In some embodiments, a method of treating a lignocellulosic material comprises degrading lignin of the lignocellulosic material with at least one fungus and hydrolyzing cellulose of the lignocellulosic material with at least one microorganism. In some embodiments, lignin is selectively degraded by the at least one fungus.

Turning now to components of methods described herein, a method described herein comprises a lignocellulosic material. Any desired lignocellulosic material not inconsistent with objectives of the present invention can be used. In some embodiments, a lignocellulosic material comprises one or more types of wood. In some embodiments, for example, wood comprises one or more types of hardwood, softwood or mixtures thereof. In some embodiments, wood comprises one or more types of genetically modified woods or plants. In some embodiments, a lignocellulosic material comprises plant leaves and/or stalks including, but not limited to, corn stover. Moreover, in some embodiments, lignocellulosic material comprises one or more grasses including, but not

limited to, switchgrass. Lignocellulosic material suitable for use in methods described herein, in some embodiments can be obtained as waste products from various applications such as timber harvesting and associated processing, agricultural harvesting and associated processing and/or landscape clearing and maintenance applications.

In some embodiments, a lignocellulosic material suitable for use in one or more methods described herein has a moisture content of at least about 10%. In some embodiments, a lignocellulosic material has a moisture content of at least about 15% or at least about 20%. A lignocellulosic material, in some embodiments, has a moisture content of at least about 30% or at least about 50%. In some embodiments, a lignocellulosic material has a moisture content ranging from about 10% to about 80% or from about 20% to about 60%. In some embodiments, moisture can be added to the lignocellulosic material prior to a treatment described herein.

In some embodiments, a lignocellulosic material is provided in particulate form. In some embodiments, for example, wood is provided in particulate form for administering a method described herein. In some embodiments, wood and/or other forms of lignocellulosic material can be chipped or ground into particulate form in preparation for administering a method described herein.

In addition to a lignocellulosic material, methods described herein comprise at least one fungus for degrading lignin of the lignocellulosic material. In some embodiments, the at least one fungus is operable to degrade lignin and cellulose of the lignocellulosic material. In some embodiments, for example, a lignin and cellulose degrading fungus comprises one or more simultaneous white rot fungi.

In some embodiments, the at least one fungus selectively degrades lignin of the lignocellulosic material. A lignin selective fungus, in some embodiments, for example, comprises a white rot fungus. Moreover, in some embodiments, the at least one fungus comprises a plurality of fungi. The plurality of fungi, in some embodiments, comprises lignin selective white rot fungi. Suitable white rot fungus or fungi can be selected according to several factors including the identity/type of lignocellulosic material provided, the moisture content and/or temperature of the lignocellulosic material and/or environment in which the method is administered. In some embodiments, suitable lignin selective white rot fungus or fungi are selected from any of the same listed in Table I herein.

Table I – Lignin Selective White Rot Fungi

Bjerkandera adusta (Polyporus adustus)
Ceriporiopsis subvermispora
Cerrena unicolor (Daedalea unicolor)
Dichomitus squalens (Polyporus anceps)
Dichomitus squalens
Dichomitus squalens FP 58543
Dichomitus squalens FP 100565
Dichomitus squalens FP 313
Ganoderma applanatum
Ganoderma lobatum FP 18692

Ganoderma lobatum FP 18679

Ganoderma oregonense

Ganoderma tsugae

Hapalopilus croceus (Polyporus croceus) FP 46228

Hapalopilus croceus FP 12503

Heterobasidion annosum (Formes annosus)

Heterobasidion annosum

Inonotus dryadeus FP 36133

Inonotus dryophilus (Polyporus dryophilus)

Inonotus dryophilus

Inonotus ludovicianus FP 18695

Inonotus rheades Strid-1076

Inonotus texanus FP 94178

Inonotus tomentosus

Ischnoderma resinosum

Laurillia sulcata (Stereum sulcatum) FP 105104

Perenniporia medulla-panis (Poria medulla-panis)

Perenniporia subacida (Poria subacida)

Perenniporia subacida FP 94346

Phellinus sp.

Phellinus fastuosus FP 21778

Phellinus nigrolimitatus (Fomes nigrolimitatus)

Phellinus nigrolimitatus

Phellinus pini
Phellinus viticola (P. isabellinus) FP 4250
Phlebia tremellosa
Scytinostroma galactinium
Xylobolus frustulatus (Stereum frustulatum)
Xylobolus frustulatus
Xylobolus subpileatus (Stereum subpileatum) FP 18502
Xylobolus subpileatus FP 12703

In some embodiments, any combination of white rot fungi of Table I can be used in a method described herein as determined according to the specific parameters of the method, including lignocellulosic material type and treatment environment.

In some embodiments, a lignin selective fungus or fungi for use in one or more methods described herein degrades at least a portion of lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material. In some embodiments, a lignin selective fungus degrades all or substantially all of the lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material. In some embodiments, degradation of the lignin provides increased access to the cellulose and/or hemicellulose of the lignocellulosic material for hydrolysis by the microorganism.

In addition to at least one lignin degrading fungus, methods described herein, in some embodiments, comprise at least one microorganism operable to hydrolyze cellulose

of the lignocellulosic material. In some embodiments, the at least one cellulose hydrolytic microorganism comprises at least one fungus. In some embodiments, for example, a microorganism comprises a brown rot fungus. The at least one microorganism, in some embodiments, comprises a plurality of fungi. In some embodiments, a plurality of fungi serving as the cellulose hydrolytic microorganism comprises brown rot fungi. Suitable brown rot fungus or fungi can be selected according to several factors including the identity/type of lignocellulosic material provided, the moisture content and/or temperature of the lignocellulosic material and/or environment in which the method is administered. In some embodiments, suitable brown rot fungus or fungi are selected from any of the same listed in Table II herein.

Table II – Brown Rot Fungi

Coniophora prasinoides
Coniophora puteana
Fomitopsis pinicola
Gloeophlyllum trabeum
Laetiporus sulphureus
Leucogyrophana arizonica
Lentinus lepidius
Piptoporus betulinus
Poria placenta
Posita placenta

Tyromyces balsameus

In some embodiments, any combination of brown rot fungi of Table II can be used in a method described herein as determined according to the specific parameters of the method, including lignocellulosic material type and treatment environment.

Alternatively, in some embodiments, a microorganism comprises one or more cellulose hydrolytic bacteria. In some embodiments, a microorganism comprises one or more cellulose hydrolytic bacteria of Table III.

Table III – Cellulose Hydrolytic Bacteria

Aerobic bacteria
Cellulomonas flavigena
C. biazotea
C. cellasea
C. fimi
C. gelida
C. cartae
C. uda
C. turbata
Bacillus brevis
B. firmus

B. lichenformis

B. pumilus

B. subtilis

B. polymyxa

B. cerus

Serrata marcescens

Heretosiphon geysericolus

Sporocytophaga myxococcoides

Streptomyces flavogriseus

Thermoonsopora curvata

Anaerobic bacteria

Acetivibrio cellulolyticus

Clostridium cellobioparum

C. papyrosolvens

C. stercorarium

C. acetobutylicum

Bacteroides succinogenes

Butyrivibrio fibrisolvens

Ruminococcus albus

R. flavefaciens

Eubacterium cellusolvens

Micromonospora ruminantium

M. propionici

In some embodiments, the at least one microorganism comprises a combination of fungus and cellulose-hydrolytic bacteria.

In some embodiments of methods described herein, a lignin degrading fungus is applied to the lignocellulosic material prior to application of the at least one microorganism. Lignin degrading fungus or fungi can be applied to the lignocellulosic material for any desired period of time prior to application of the at least one cellulose hydrolytic microorganism. In some embodiments, for example, a lignin degrading fungus is applied to the lignocellulosic material for a time period sufficient to degrade a desired amount of lignin.

The time period over which a lignin degrading fungus is applied to a lignocellulosic material can be dependent on several factors including the identity of the fungus, identity of the lignocellulosic material, amount of the lignocellulosic material and/or the surrounding environmental conditions. In some embodiments, for example, a lignin degrading fungus is applied to the lignocellulosic material for a time period of at least about 1 week. In some embodiments, a lignin degrading fungus is applied to the lignocellulosic material for a time period of at least about 2 weeks or at least about 3 weeks. In some embodiments, a lignin degrading fungus is applied to the lignocellulosic material for a time period ranging from about 1 week to about 5 weeks. In some embodiments, a lignin degrading fungus is applied to the lignocellulosic material for a time period ranging from about 2 weeks to about 4 weeks. In some embodiments, a

lignin degrading fungus is applied to the lignocellulosic material for a time period less than about 1 week.

In some embodiments, a lignin degrading fungus or fungi is operable to degrade at least about 1 weight percent of the lignin content of the lignocellulosic material. In some embodiments, a lignin degrading fungus or fungi degrades at least about 5 weight percent or at least about 10 weight percent of the lignin content of the lignocellulosic material. In some embodiments, a lignin degrading fungus or fungi degrades at least about 25 weight percent or at least about 50 weight percent of the lignin content of the lignocellulosic material. In some embodiments, a lignin degrading fungus or fungi degrades from about 1 weight percent to about 90 weight percent of the lignin content of the lignocellulosic material. In some embodiments, a lignin degrading fungus or fungi degrades from about 5 weight percent to about 75 weight percent of the lignin content of the lignocellulosic material. In some embodiments, a lignin degrading fungus or fungi degrades from about 10 weight percent to 60 weight percent of the lignin content of the lignocellulosic material. Moreover, as described herein, a lignin degrading fungus or fungi, in some embodiments, can selectively degrade lignin of the lignocellulosic material in any of the foregoing amounts without degrading or substantially degrading the cellulose content of the lignocellulosic material.

In some embodiments wherein a lignin degrading fungus is applied to the lignocellulosic material, the lignocellulosic material is sterilized or substantially sterilized prior to application of the at least one microorganism. In some embodiments, the lignocellulosic material treated with the at least one lignin degrading fungus can be sterilized with steam or through various autoclaving techniques. In some embodiments,

for example, a lignin selective white rot fungus is applied to the lignocellulosic material for a desired period of time. The lignocellulosic material is sterilized or substantially sterilized prior to application a microorganism, such as a brown rot fungus, for the hydrolysis of cellulose of the lignocellulosic material.

Alternatively, in some embodiments, the lignocellulosic material is not sterilized prior to application of the at least one microorganism such that the lignin degrading fungus and the cellulose hydrolytic microorganism exist simultaneously on the lignocellulosic material.

In some embodiments, the at least one microorganism can be applied to the lignocellulosic material prior to application of the at least one lignin degrading fungus. In some embodiments, the lignocellulosic material comprising the microorganism is sterilized or substantially sterilized prior to application of a lignin degrading fungus. In some embodiments, the lignocellulosic material is not sterilized prior to application of a lignin degrading fungus such that the lignin degrading fungus and cellulose hydrolytic microorganism exist simultaneously on the lignocellulosic material.

Additionally, in some embodiments, a lignin degrading fungus and a cellulose hydrolytic microorganism are applied to the lignocellulosic material simultaneously or substantially simultaneously.

A cellulose hydrolytic microorganism can be applied to the lignocellulosic material for any desired period of time. In some embodiments, for example, a cellulose hydrolytic microorganism is applied to the lignocellulosic material for a time period sufficient to hydrolyze a desired amount of cellulose and/or hemicellulose.

The time period over which a cellulose hydrolytic microorganism is applied to a lignocellulosic material can be dependent on several factors including the identity of the microorganism, identity of the lignocellulosic material, amount of the lignocellulosic material and/or the surrounding environmental conditions. In some embodiments, for example, a cellulose hydrolytic microorganism is applied to the lignocellulosic material for a time period of at least about 1 week. In some embodiments, a cellulose hydrolytic microorganism is applied to the lignocellulosic material for a time period of at least about 2 weeks or at least about 3 weeks. In some embodiments, a cellulose hydrolytic microorganism is applied to the lignocellulosic material for a time period ranging from about 1 week to about 5 weeks. In some embodiments, a cellulose hydrolytic microorganism is applied to the lignocellulosic material for a time period ranging from about 2 weeks to about 4 weeks. In some embodiments, a cellulose hydrolytic microorganism is applied to the lignocellulosic material for a time period less than about 1 week.

In some embodiments, the cellulose hydrolytic microorganism is operable to depolymerize cellulose and/or hemicellulose of the lignocellulosic material into shorter polysaccharides, oligosaccharides or monomeric sugars or mixtures thereof. In some embodiments, a cellulose hydrolytic microorganism depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length of up to about 1000. In some embodiments, a cellulose hydrolytic microorganism depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length of up to about 700 or up to about 500. In some embodiments, a cellulose hydrolytic microorganism

depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length of up to about 400 or up to about 300.

In some embodiments, a cellulose hydrolytic microorganism depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length ranging from about 10 to about 1000. In some embodiments, a cellulose hydrolytic microorganism depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length ranging from about 50 to about 750. In some embodiments, a cellulose hydrolytic microorganism depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length ranging from about 100 to about 500. In some embodiments, a cellulose hydrolytic microorganism depolymerizes cellulose of the lignocellulosic material into polysaccharides and/or oligosaccharides having an average chain length ranging from about 200 to about 400.

In some embodiments, any cellulose hydrolytic microorganism described herein can depolymerize cellulose of the lignocellulosic material to produce polysaccharides, oligosaccharides or mixtures thereof having any of the foregoing average chain lengths. In some embodiments, for example, brown rot fungi described herein can depolymerize cellulose of the lignocellulosic material to produce polysaccharides, oligosaccharides or mixtures thereof having any of the foregoing average chain lengths.

In some embodiments, degradation of lignin material by the lignin degrading fungus facilitates access to cellulose of the lignocellulosic material for the cellulose hydrolytic microorganism. As a result, the lignin degrading fungus or fungi and cellulose

hydrolytic microorganism, in some embodiments, work in conjunction to increase cellulose depolymerization of the lignocellulosic material.

In some embodiments, a lignin degrading fungus and/or a cellulose hydrolytic microorganism is applied to a lignocellulosic material in any suitable manner known to one of skill in the art. In some embodiments, for example, a lignin degrading fungus and/or a cellulose hydrolytic microorganism is applied to a lignocellulosic material by liquid phase techniques. In some embodiments, a lignin degrading fungus and/or cellulose hydrolytic microorganism is applied to a lignocellulosic material by contacting the lignocellulosic material with a lignocellulosic material containing the lignin degrading fungus and/or cellulose hydrolytic microorganism.

Embodiments of methods described herein contemplate any combination of lignin degrading fungus or fungi and cellulose hydrolytic microorganisms. In some embodiments, for example, methods described herein contemplate any combination of white rot fungi of Table I with brown rot fungi of Table II and/or cellulose hydrolytic bacteria of Table III.

Moreover, in some embodiments of methods described herein, cellulose and/or hemicellulose of the lignocellulosic material can be further depolymerized by one or more cellulase enzymes into monomeric sugars, including various hexoses, pentoses or mixtures thereof. In some embodiments, cellulose of the lignocellulosic material is treated with one or more endocellulases, exocellulases, cellobiases, oxidative cellulases and/or cellulose phosphorylases. In some embodiments, for example, a cellulose of the lignocellulosic material can be further depolymerized with one or more exoglucanases,

endoglucanases, β-glucosides and/or oxidative enzymes to produce glucose monomeric units.

In some embodiments, treatment of a lignocellulosic material with at least one lignin degrading fungus and at least one cellulose hydrolytic microorganism provides an increased amount of cellulose in a morphology for facile depolymerization by one or more cellulases into monomeric sugars. As a result, methods described herein, in some embodiments, can assist in realizing increased conversion of lignocellulosic biomass into monomeric sugars for various uses including feedstocks and ethanol production.

In another aspect, methods of producing ethanol are described herein. In some embodiments, a method of producing ethanol comprises providing a lignocellulosic material, degrading lignin of the lignocellulosic material with at least one fungus, hydrolyzing cellulose of the lignocellulosic material with at least one microorganism, further hydrolyzing the cellulose with one or more cellulase enzymes into one or more fermentable sugars and fermenting the one or more sugars into ethanol.

In some embodiments, lignin of the lignocellulosic material is selectively degraded by the at least one fungus. In some embodiments of methods of producing ethanol, the at least one fungus comprises a white rot fungus. In some embodiments, the at least one fungus comprises a plurality of fungi. The plurality of fungi, in some embodiments, comprises white rot fungi.

In some embodiments, the at least one cellulose hydrolytic microorganism of a method of producing ethanol comprises at least one fungus. In some embodiments, for example, a cellulose hydrolytic microorganism comprises a brown rot fungus. The at least one microorganism, in some embodiments, comprises a plurality of fungi. In some

embodiments, a plurality of fungi serving as the cellulose hydrolytic microorganism comprises brown rot fungi. Alternatively, a microorganism, in some embodiments, comprises one or more cellulose hydrolytic bacteria. In some embodiments, the at least one microorganism comprises a combination of fungus and cellulose hydrolytic bacteria.

In some embodiments of producing ethanol, any lignin degrading fungus and cellulose hydrolytic microorganism described herein can be used.

In some embodiments of producing ethanol, a lignin degrading fungus is applied to the lignocellulosic material prior to application of the at least one cellulose hydrolytic microorganism. In some embodiments, the microorganism is applied to the lignocellulosic material prior to application of a lignin degrading fungus. In some embodiments, a lignin degrading fungus and a microorganism are applied to the lignocellulosic material simultaneously or substantially simultaneously.

In some embodiments of producing ethanol wherein a lignin degrading fungus is applied to the lignocellulosic material, the lignocellulosic material is sterilized or substantially sterilized prior to application of the at least one cellulose hydrolytic microorganism. In some embodiments, for example, a lignin selective white rot fungus is applied to the lignocellulosic material for a desired period of time. The lignocellulosic material is subsequently sterilized or substantially sterilized prior to application of a microorganism, such as a brown rot fungus, for the hydrolysis of cellulose of the lignocellulose material.

In some embodiments, the lignocellulosic material is not sterilized prior to application of the at least one microorganism such that the lignin degrading fungus and

the cellulose hydrolytic microorganism exist simultaneously on the lignocellulosic material.

In some embodiments, a lignin selective fungus or fungi degrades at least a portion of lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignin cellulosic material. In some embodiments, a lignin selective fungus or fungi degrades all or substantially all of the lignin of the lignocellulosic material without degrading or substantially degrading cellulose of the lignocellulosic material.

In some embodiments, degradation of the lignin provides increased access to the cellulose and/or hemicellulose of the lignocellulosic material for hydrolysis by the microorganism and one or more cellulase enzymes. Hydrolysis of cellulose by the microorganism and one or more cellulase enzymes, including those described herein, can produce sugars suitable for fermentation into ethanol by techniques known to one of skill in the art. In some embodiments of producing ethanol, monomeric sugars produced according to methods described herein comprise hexoses, pentoses and/or mixtures thereof. In some embodiments, hexoses comprise glucose, mannose or galactose or mixtures thereof. In some embodiments, pentoses comprise xylose or arabinose or mixtures thereof.

In another aspect, bioreactors for treating lignocellulosic material are described herein. In some embodiments, a bioreactor comprises a container for storing lignocellulosic material and apparatus for introducing at least one lignin degrading fungus and at least one cellulose-hydrolytic microorganism to the lignocellulosic material in the container. In some embodiments, a lignin degrading fungus and cellulose

hydrolytic microorganism introduced to the lignocellulosic material through the apparatus of the bioreactor can comprise any lignin degrading fungus and cellulose hydrolytic microorganism described herein.

In some embodiments, a container of a bioreactor described herein has a volume sufficient to hold at least 1 ton of lignocellulosic material. In some embodiments, a bioreactor container has a volume sufficient to at least 2 tons or at least 3 tons of lignocellulosic material. In some embodiments, a bioreactor container has a volume sufficient to at least 4 tons or at least 5 tons of lignocellulosic material.

In some embodiments, apparatus for introducing at least one lignin degrading fungus to the lignocellulosic material comprises a fluid transport system with one or more injection points for liquid phase application of the at least one lignin degrading fungus. Similarly, in some embodiments, apparatus for introducing at least one cellulose hydrolytic microorganism to the lignocellulosic material comprises a fluid transport system with one or more injection points for liquid phase application of the at least one cellulose hydrolytic microorganism.

Moreover, in some embodiments, a bioreactor described herein further comprises one or more agitators for mixing or mechanically agitating the lignocellulosic material in the container. Additionally, in some embodiments, a bioreactor described herein comprises apparatus suitable for autoclaving or otherwise sterilizing the lignocellulosic material in the container, such as apparatus for steam treating the lignocellulosic material.

In some embodiments, a bioreactor described herein further comprises climate control apparatus for regulating the temperature and moisture content of the lignocellulosic material and/or surrounding environment. In some embodiments, a

bioreactor comprises water spraying apparatus to control the moisture content of the lignocellulosic material as well as heating and cooling apparatus for controlling temperature of the lignocellulosic material and surrounding environment.

Embodiments of compositions and methods described herein are further illustrated by the following non-limiting examples.

EXAMPLE 1

Treatment of Lignocellulosic Material

A lignocellulosic material was treated in accordance with one embodiment of a method described herein as follows:

I. Fungal isolates, culture conditions, and wood

Fresh cut *Liriodendron tulipifera* (Tulip Poplar) wood chips (≈ 3 x 3 x 0.5 cm) were collected from a hardwood lumber mill and stored at 4°C until use. The wood was a mixture of earlywood and latewood chipped from duramen without orientation. The chips were visually inspected to verify the homogeneity of all the samples before treatment (defect wood removed). *Ceriporiopsis subvermispora* FP-90031-sp (a lignin-selective white rot fungus), and *Postia placenta* Mad-698-R (a brown rot fungus) were obtained from U.S.D.A. Forest Products Laboratory, Madison, Wisconsin, USA. The fungi were independently cultured in malt extract agar (MEA) and incubated at 28°C for 7-10 days. A fungal plug from the malt extract agar plate was then placed in 500 ml malt extract liquid medium and incubated at 28°C for 7-10 days.

All treatments were conducted in triplicate. For each treatment, 1 gram (oven dry weight) of wood chips was placed in 20 ml scintillation vials and distilled water was added to increase moisture content to 70%. The loosely capped vials were then steam sterilized for 30 minutes. The malt extract liquid fungal cultures were vigorously shaken for one minute before use, and 0.2 ml was used to aseptically inoculate each wood chip treatment vial. The controls were also sterilized and 0.2 ml of media from the malt extract cultures was sterile filtered and added to each sterile control vial. The vials were then incubated for 30 days at 28°C for optimal delignification and depolymerization. After the 30 day colonization period, the vials designated for two-stage biopulping treatment were steam sterilized for 30 minutes and then inoculated with the second fungal species, using the same inoculation volume and sterile media control as before. The two-

stage treatment vials were then incubated for an additional 30 days at 28°C. The treatment vials were then oven dried 24 hours at 104°C.

A summary of the *Liriodendron tulipifera* treatment classes is provided in Table IV.

Table IV - *Liriodendron tulipifera* treatment classes

Treatment	Stage I	Stage 2
1 - Untreated/Sterile	Sterilize (30 min)	None
2 - Single Stage White Rot	Sterilize followed by application of *Ceriporiopsis subvermispora* FP-90031-sp	None
3 - Untreated/Sterile	Sterilize (30 min)	Sterilize (30 min)
4 - Single Stage Brown Rot	Sterilize followed by application of *Postia placenta* Mad-698-R	None
5 - Two Stage White Rot	Sterilize followed by	Sterilize followed by

followed by Brown Rot	application of *Ceriporiopsis subvermispora* FP-90031-sp	application of *Postia placenta* Mad-698-R
6 - Two State Brown Rot followed by White Rot	Sterilize followed by application of *Postia placenta* Mad-698-R	Sterilize followed by application of *Ceriporiopsis subvermispora* FP-90031-sp

II. Chemical Analysis

Gravimetric determination of Klason lignin content of the untreated/sterile controls and fungi treated samples were performed using previously described microanalytical techniques. (Yokohama et al., *Journal of Agricultural and Food Chemistry*, 50, 1040-1044; Yeh et al., *Journal of Agricultural and Food Chemistry*, 53, 1435-1439).

Klason lignin was prepared from extractive free wood meal. For each sample, 300 mg of oven dry weight wood and 3 mL of 72% sulfuric acid was placed in a 90 mL pressure tube. The sample was then stirred with a glass rod every 15 min for 60 min. The acid was then diluted to 4% concentration by addition of 84 mL of deionized water. The pressure tubes were then capped and autoclaved for one hour at 121°C,cooled overnight at 4°C, and filtered using a sintered glass filter (medium pore size). Acid insoluble lignin was washed with deionized water and dried at 104°C before weighing. Klason lignin contents were recorded as a percentage of sample mass.

III. Enzymatic Hydrolysis

Enzymatic hydrolysis was performed on ground material (40 mesh particle size) from triplicate treatments and controls using a modified method outlined in Shi et al., *Biomass and Bioenergy*, 33, 88-96, 2009, including a 1:1.75 mixture of cellulase (22 FPU/g of substrate) (Celluclast 1.5L, Sigma Co.) and β-glucosidase (Novozyme 188, Sigma Co.) and a 3% biomass loading volume (3 replicate samples and 3 replicate controls without enzyme per biopulping treatment). Samples were shaken at 50°C for 72 hours then centrifuged for 10 minutes at 3000 rpm. Aliquots of supernatants were filtered a 0.22 μm filter. Samples were analyzed by HPLC using electrochemical detection and a Carbo-Pac10 carbohydrate column (Dionex) (Lee et al., *Journal of Bioscience and Bioengineering*, 106, 162-167). The mobile phase was 3 mM NaOH, which was circulated with a flow rate of 0.2 mL/min. Quantification and identification of peaks were performed using dilutions of arabinose, galactose, glucose, xylose, and mannose stock standards. All peaks eluted within 45 min. Glucose contents were normalized to controls without enzymes.

Figure 1 illustrates the lignin content of wood of control treatment 1 in comparison with treatments 2, 4 and 5 of Table IV. The single stage white rot fungus of treatment 2 removed 1.5% of the lignin from the wood while the single stage brown rot fungus did not remove any lignin content from the wood. The two-stage process of treatment 5 removed 2% of the lignin from the wood. Error bars of Figure 1 = 1 SE.

Figure 2 illustrates percent biomass converted to glucose according to treatments 1, 2, 3, 5 and 6 of Table IV. As illustrated in Figure 2, the two stage process of treatment

5, according to one embodiment of a method described herein comprising application of a lignin degrading fungus followed by a cellulose hydrolytic fungus, resulted in the highest percent conversion of lignocellulosic biomass into soluble glucose. Moreover, the two stage process of treatment 6, according to one embodiment of a method described herein comprising application of a cellulose hydrolytic fungus followed by a lignin degrading fungus, resulted in the second highest percent conversion of lignocellulosic biomass to soluble glucose. Error bars of Figure 2 = 1SE.

Various embodiments of the invention have been described in fulfillment of the various objectives of the invention. It should be recognized that these embodiments are merely illustrative of the principles of the present invention. Numerous modifications and adaptations thereof will be readily apparent to those skilled in the art without departing from the spirit and scope of the invention.

That which is claimed is:

CLAIMS

1. A method of treating a lignocellulosic material comprising:
degrading lignin of the lignocellulosic material with at least one fungus; and
hydrolyzing cellulose of the lignocellulosic material with at least one microorganism.

2. The method of claim 1 wherein the at least one fungus comprises a white rot fungus.

3. The method of claim 1, wherein the at least one fungus comprises a plurality of fungi.

4. The method of claim 3, wherein the plurality of fungi comprise white rot fungi.

5. The method of claim, wherein the lignin is selectively degraded with the at least one fungus.

6. The method of claim 5, wherein the at least one fungus comprises white rot fungus.

7. The method of claim 6, wherein the fungus comprises *Ceriporiopsis subvermispora, Phlebia tremellosa* or *Scytinostroma galactinium*.

8. The method of claim 1, wherein the microorganism comprises at least one fungus.

9. The method of claim 8, wherein the at least one fungus comprises a brown rot fungus.

10. The method of claim 2, wherein the at least one microorganism comprises a brown rot fungus.

11. The method of claim 8, wherein the microorganism comprises a plurality of fungi.

12. The method of claim 11, wherein the fungi comprises brown rot fungi.

13. The method of claim 1, wherein the microorganism comprises one or more bacteria.

14. The method of claim 1, wherein at least 5 weight percent of the lignin is degraded.

15. The method of claim 1, wherein at least 10 weight percent of the lignin is degraded.

16. The method of claim 1, wherein the average chain length of the hydrolyzed cellulose is less than about 1000.

17. The method of claim 1, wherein the average chain length of the hydrolyzed cellulose ranges from about 100 to about 400.

18. The method of claim 1, wherein the at least one fungus is applied to the lignocellulosic material prior to application of the at least one microorganism.

19. The method of claim 18 further comprising sterilizing the lignocellulosic material prior to application of the microorganism.

20. The method of claim 1 further comprising hydrolyzing the cellulose with one or more cellulases to produce monomeric sugars.

21. A method of producing ethanol comprising:
providing a lignocellulosic material;
degrading lignin of the lignocellulosic material with at least one fungus;

hydrolyzing cellulose of the lignocelluosic material with at least one microorganism;

further hydrolyzing the cellulose with one or more cellulase enzymes into one or more fermentable sugars;

fermenting the one or more sugars to produce ethanol.

22. The method of claim 21, wherein the one or more fermentable sugars comprise a hexose, pentose or mixtures thereof.

23. The method of claim 21, wherein the lignin is selectively degraded with the at least one fungus.

24. The method of claim 23, wherein the at least one fungus is a white rot fungus.

25. The method of claim 24, wherein the at least one microorganism comprises a brown rot fungus.

ABSTRACT

In one aspect, methods of treating lignocellulosic materials are described herein. In some embodiments, a method of treating a lignocellulosic material comprises degrading lignin of the lignocellulosic material with at least one fungus and hydrolyzing cellulose of the lignocellulosic material with at least one microorganism.

APPENDIX B: DEVELOPMENT OF A NOVEL COST-EFFECTIVE SPECTROGRAPHIC (RAMAN AND FTIR) TOOL FOR EVALUATING WOODY FEEDSTOCKS DURING BIOPULPING

The following appendix is currently being written into a manuscript for submission to the scientific journal *Journal of Agricultural and Food Chemistry*.

1. Introduction

There are many different kinds of woody plant materials that may be used for ethanol production (e.g. different wood types, plant stems and grasses), but managers require cost-effective methods to determine initial suitability and breakdown efficiency during processing. While traditional analytical chemistry, NMR, and SEM methods are accurate and reliable, they are also expensive and time-consuming. New technologies show great promise for rapid, less costly examination of biomass components in plant materials. Raman spectroscopy and Fourier Transform Infrared spectroscopy (FTIR) are novel methods that allows quick chemical analysis of woody sample properties using only optical (light) measurements (Yamada et al. 2006, Fackler et al. 2007). However, in order to be properly interpreted, light spectra detected must be directly correlated with traditional chemical analyses on the same materials.

We have directly correlated our traditional analytical chemistry measurements of lignin and sugar content with Raman and FTIR spectra collected from pre-and post-treatment feedstocks described in Chapters 1,2 and in pilot treatments at the Catawba County EcoComplex. These correlations have been used to construct much-needed models for

rapid analyses of sugar availability in various feedstocks, both before and during biopulping. These innovative model will provide industrial managers with a rapid, less costly method for examining potential feedstocks for ethanol production suitability as well as evaluating batch quality, without requiring expensive chemical analyses. Here we describe two representative models developed during this project.

2. Results

FTIR Spectroscopy

Figure B.1 represents the relationship between the actual lignin to cellulose ratio that has been determined by gravimetric analysis versus the predicted lignin to cellulose ratio. The predicted values were generated based on a linear regression model produced by partial least squares regression conducted in JMP 9. Spectroscopic lignin values were determined by integrating the area under the peak at 1508 cm^{-1}. Spectroscopic cellulose values were determined by integrating the areas under the peaks at 1748cm^{-1}, 1135cm^{-1}, and 898cm^{-1}.

The samples measured were part of an experiment consisting of three replicates for each replicate. The replicates for each treatment were averaged to produce a single value. This was done for both gravimetric analysis and spectral measurements. Statistical measures (PLS) were applied to the mean spectral values and thus the predicted values were generated from the replicate averages.

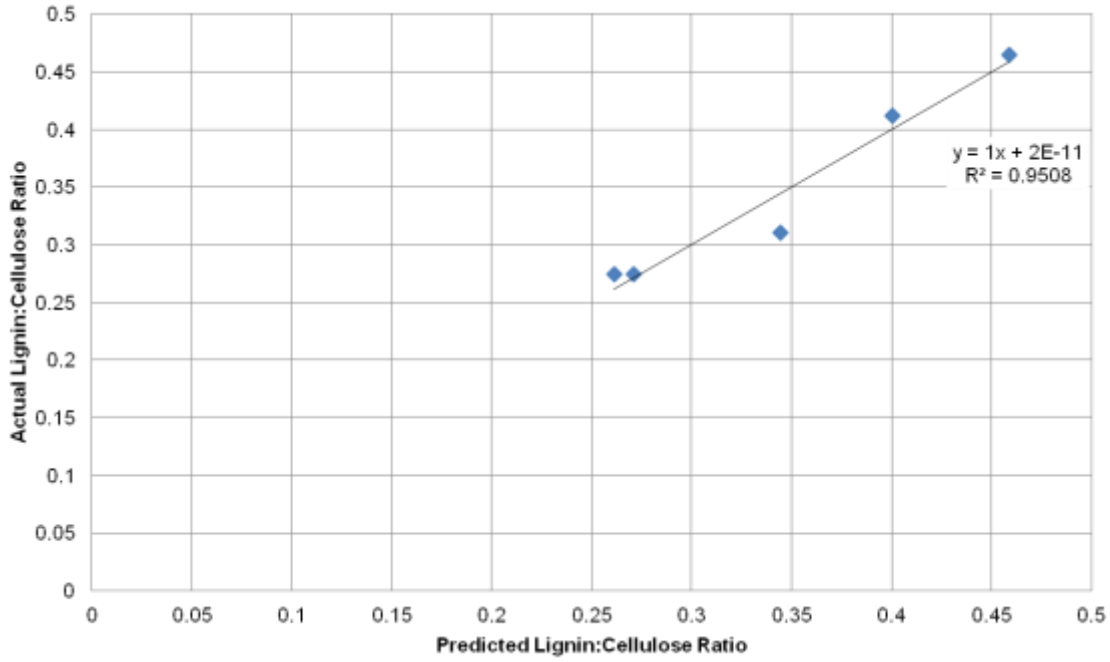

Figure B.1: Example of wood sample lignin and cellulose contents predicted from FTIR spectra.

Raman Spectroscopy

Figure B.2 shows the predicted lignin to cellulose ratio as predicted by Raman spectroscopy versus the actual lignin to cellulose ratio as determined by gravimetric analysis. The same values for actual lignin to cellulose ratios were used in both the Raman and IR figures. Raman predictions were made using the replicate averages of each treatment as with FT-IR spectroscopy. Peak areas were also used to quantify the lignin and cellulose concentrations within the sample. The peak for lignin was found at 1600 cm^{-1} and the cellulose peak was between 1010

and 1200 cm^{-1}.

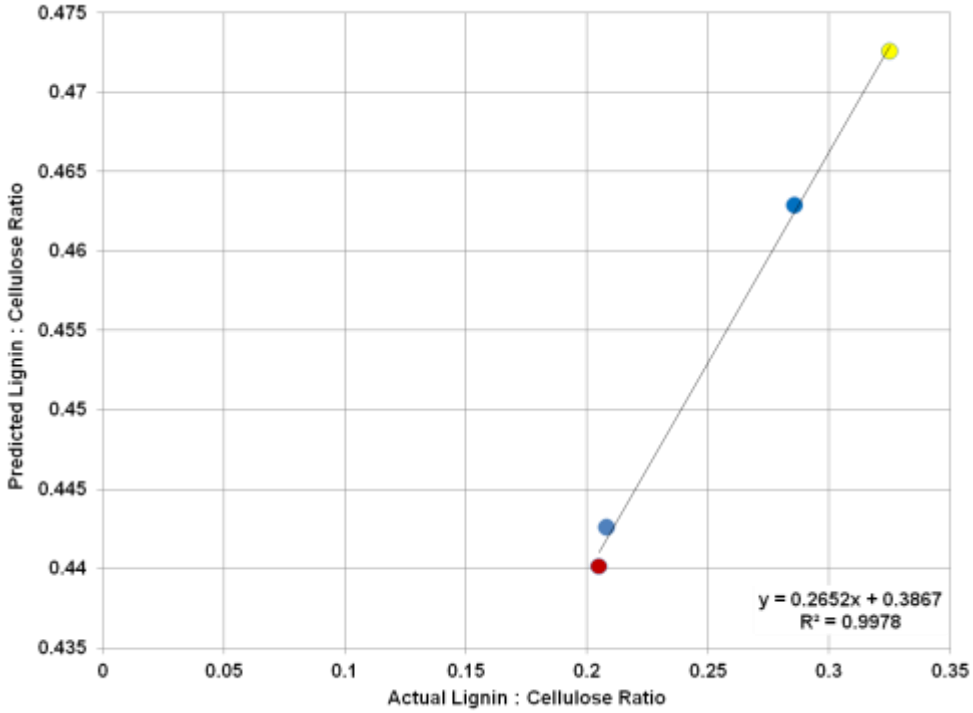

Figure B.2: Example of wood sample lignin and cellulose contents predicted from FTIR spectra.

APPENDIX C: DESIGNS FOR THE CATAWBA COUNTY ECOCOMPLEX CELLULOSIC ETHANOL PRODUCTION FACILITY

The following appendix contains tanks designed by the author and engineers at Mixer Direct (Louisville, KY) for installation into the Catawba County EcoComplex Ethanol Production Facility. The two tank system is designed to utilize all types of biopulped lignocellulosic biomass produced onsite for the production of ethanol. The system contains an 500 gallon enzymatic hydrolysis and a 600 gallon fermentation/distillation tank. This system will be installed and operational in late 2013.